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and Breast Cancer Etiology/Outcome

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13. ABSTRACT (Maximum 200 Words)

Positive Estrogen Receptor alpha (ER) status correlates with a reduced incidence of breast cancer recurrence in the first years after resection of tumors, and predicts a favorable response to adjuvant anti-estrogens. ER-protein in breast tumors increases with patient age. The project develops novel antibody reagents to probe the expression of ER from alternate promoters taking advantage of short peptides encoded in regions of the ER mRNAs that are promoter specific and not shared. We hope that the short transcript specific peptides will act as 'Biomarkers' of promoter use.

In the second year we have developed a new set of polyclonal antisera to one of the short upstream peptides and these are to be tested in immunohistochemical assays for ability to assess promoter use. A set back has been our failure to develop successful monoclonal antibodies, though the primed mice had good immune responses.

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Introduction

The project develops novel antibody reagents to probe the expression of Estrogen Receptor alpha (ER) from alternate promoters taking advantage of short peptides whose starts are encoded in regions of the ER mRNAs that are promoter specific and not shared (Table1). ER mRNA is derived from two main promoters. Regulation of ER expression from alternate promoters could be important in breast cancer development and progression, as they appear to be under different controls. Regulation of the promoters could be a targeted in order manipulate ER status of tumors.

Positive ER status correlates with a reduced incidence of breast cancer recurrence in the first years after resection of tumors, and predicts a favorable response to adjuvant anti-estrogens and ER-protein in breast tumors increases with patient age (1). Hayashi et al (2) studied the ER protein level, total mRNA level, and relative promoter usage in ten ER positive breast tumors. They saw a positive correlation of breast tumor ER-protein expression and relative usage of the distal promoter after menopause. We think their data suggests the age related increase in tumor ER levels is partly due to a specific increase in distal promoter use, indicating that controls on the promoters are different. However, the RT-PCR-based assays for alternate transcripts are time consuming and difficult and, they are not a feasible clinical test. We are attempting to develop antibody-based assays exploiting the short peptides encoded in upstream open reading frames (uORFs) of the ER mRNAs that are promoter specific.

Task 1. Polyclonal Antisera to Upstream peptides of the alternate Estrogen receptor transcripts

Year 1:

Synthesize peptides.
Initiate generation of Polyclonal antisera in rabbits.
Characterize antisera in Western blots using phased program described in plan.
Evaluate need to modify peptide immunogens based on specificity and sensitivity of preliminary results

Year 2:

Apply antisera to immuno-histochemistry assay (IHA) format on cells.
Obtain tumor sections.
Apply antisera to tumor sections in IHA format.

We are running somewhat behind in Task 1 as the first generation polycolanal antisera were either ineffective (anti- prox-peptide) or not strong enough for applications to tissue sections (anti-distal-peptide). However new antisera against the proximal uORF peptide are encouraging. These new antisera are being evaluated and we have taken the opportunity of this report to crystallise our thoughts on how to test the new antisera. We have been successful in obtaining breast tumor blocks for future analysis.

Polyclonals: Rabbit immune reagents against the petide of the distal ER-ORF

We have prepared an rabbit antiserum, Y747, against the distal ER promoter uORF peptide. This was described in the prior annual report. This antisera had a heavy background against HeLa cells. We therefore tried two methods two reduce the background: We attempted immunopurification on a column of a distal peptide-maltose binding protein (MBP) fusion and immuno depletion on a column of HeLa protein extract. The first method was more effective.

The background associated with the antiserum was reduced by immunopurification on a column of recombinant MBP with the distal peptide incorporated in frame as the C- terminus (preparation was described in the prior report). Judging from the behavior of the immunopurified serum we think the

major result of this process was the eliminations of non-specific immunoglobulin and the background was reduced because we were adding less material that the detection system (second antibody) could interact with in western blots. Our first test of the Y747 antisera was against the recombinant MBP fusion protein (as reported last year). The follow up to this was work with HeLa cells transfected with distal-peptide-GFP fusion constructs . The model system has two advantages: first we have the confidence that the desired protein is present because of the option of probing with anti-GFP antisera. Second the 22000 Da fusion protein is a convenient size and binds to membrane whereas the small 2000 Da peptide alone does not.

The fusion of GFP and the distal peptide was expressed from a construct we used in early studies (see original grant application) where the uORF was fused 5' to that for GFP i.e. at the N-terminus of GFP (see Fig. 15, A&B). The Y747 antiserum failed to detect the GFP fusion though the product was detectable (though the GFP fraction) with an anti-GFP antiserum. The first thought was that the antiserum was too weak for the application. A second thought was that the original coupling of peptide to carrier streptavidin was through the N-terminus of the peptide and that was similar to configuration in the MBP fusion (Fig. 15 D) while in the original GFP-peptide construct the link was from the other end of the peptide to the N-terminus of GFP and potentially constrained the C-terminus of the peptide with the risk of conformational problems in analysis.

We therefore made new GFP fusion constructs for both the distal and proximal constructs (the latter may be needed in future expt) where the uORF peptides are C-terminal extensions of GFP (Fig.1C). Blots in Fig. 2 are of cell extracts of cells transfected transiently with GFP constructs. All of the transfected cell populations show a band with a GFP antiserum. The size varies depending on the peptide ORF (if any) fused to the GFP ORF and on the length of any intervening sequences that become part of the final ORF, this is different for C- and N-terminal fusions. We were able to detect signal with the anti-distal Y747 anti-serum in addition to GFP in western immunoblots of cell extracts of cells transfected with the distal uORF peptide C-terminal to GFP (tracks 1&2 in Fig. 2) but not when it was N-terminal to GFP (track 3) construct. However, this is really too weak for any routine use and this was not encouraging for future detection of the free peptide.

In an attempt boost detection we tried using the more sensitive 'Femto' chemiluminescent detection system from Pierce in place of the usual 'Pico Supersignal'. The femto detection system is more sensitive, but with more background problems. Following instructions we reduced the amount of added antibody but this limited detection. Hence we effectively get the same limits on detection. The limits on detection appear to be set by the affinity of the antiserum not some aspect of the detection system. Recently we have been using the BiaCore to evaluate sera and a clear point is that the immunopurified Y747 antiserum fraction is easily washed from the BiaCore chip. This is indicative of low affinity, but only became a meaningful observations when contrasted with the data for the new antisera against proximal peptide (Fig. 4, see below).

The Y747 antiserum seems unlikely to be usable against the free distal peptide in ER -positive cells, though it will be tested in models for free peptide expression with immuno-histochemical Detection. We have to establish this new model for use with the new rabbit polyclonal anti-sera again the ER proximal uORF peptide. We are proposing to schedule the distal peptide for use in generating monoclonals (see Task 2).

Polyclonals: New Rabbit Polyclonal Antisera to ER Proximal promoter uORF peptides

We made a second attempt to developed polycolnal antisera to the proximal peptide based on experience with rabbit polyclonals and with mouse immunization (see Task 3). The two immunogens were a raw full length prox peptide carrying the unique and shared regions *without* linkage to any carrier and a fusion of the prox-peptide to the C-terminus of maltose binding protein (MBP). In the latter case the MBP may act as 'carrier' possibly altering presentation to the immune system. Rabbits were immunized using complete Freund's adjuvant initially. Follow up boosting was in incomplete Freund's

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adjuvant as approved by our IACUC.

We have done the primary immunization and have been boosted twice, at monthly intervals. Test bleeds were done prior to any immunization and two weeks after each immunization in incomplete Freund's adjuvant.

Rabbits serum from the prebleed (bleed O) and 2 subsequent bleeds (bleeds 1 and 2) were analyzed on the BiaCore. Data with serum from the second bleed is shown in Fig.3, data is shown for the pre-regenerative wash phase, ie for higher affinity interactions, though data is largely similar in the binding phase. BiaCore analysis was similar to that for mouse sera (Task 3): some of original BiaCore traces are shown (see Fig. 5) for that data.

The rabbits immunized with the raw peptide (unconjugated synthetic peptide) showed the strongest and most specific signal in the BiaCore assay. There was clear discrimination for the prox peptide sector over distal sector with the sera from rabbits receiving the raw peptide. Note that BiaCore signal was limited for rabbits Y936 and Y941 that received the MBP fusion protein, and discrimination between distal and proximal peptide bound to BiaCore sectors was poor.

A more comprehensive data set is shown for serum fromY935 in Fig. 4. Y935 was one of the rabbits immunized with free peptide. The signal from interaction between the proximal peptide on the chip and serum of rabbit Y935 clearly was aquired after immunization. An important point is that the immunopurified antiserum Y747 against distal ER uORF peptide is shown for comparison in Fig 4: Y747 shows specificty for the distal peptide sector as expected *but* it is largely washed out in the pre-regenateve phase, ie Y747 antiserum has lower affinity.

Western blot analysis of rabbit Y935 is shown in Fig. 7. This analysis employed serum from the second test bleed. Rabbit Y935 was immunized with complete ER proximal promoter uORF peptide alone (no carrier) and gives a strong signal in the BiaCore. There is discrimination between recombinant prox-peptide MBP fusion at 20 ng of MBP vs the MBP-prox-peptide fusion in the second bleed but there is a precipitous decline in the specific signal at 4 ng protein. We cannot easily, or at least meaningfully, test the serum from rabbits (Y941 and Y936) immunized with MBP fusions in this assays as the rabbits have antibodies against the MBP protein potions of the protein we are including on gels. However, in the lower panel of Fig. 7 we show a blot incubated with serum from rabbit Y941. This serum shows a strong interaction with both MBP and MBP-prox fusion *at all levels* of protein tested. We had attempted to immuno-deplete the serum of anti-MBP antibodies on an MBP column, but this failed to eliminate interaction with MBP. The main point made by including this panel is to contrast behavior seen with the Y935 antiserum in the upper panel which does function in a western blot, but with limited sensitivity. Note that in the sections below we describe how Y935 does NOT detect GFP–prox peptide fusions in transfected cells, in contrast to serum Y941 raised against the recombinant peptide-MBP fusion.

One of our tests for antisera is detection of GFP-fusion proteins in extracts from transfected cells. This assay allows a measure of specificity for the ER distal/proximal uORF peptides versus general mammalian cell proteins. The fusions proteins run at about 20 kDa in the gels and blots which makes them easy to analyze. Expression levels are likely higher than for the actual peptide (in an ER expressing cell) as expression in the transfected cells is from a strong viral promoter, though only about 20% of cells were transfected in this experiment. The actual uORF proteins are ~ca 2 kDa which is too small for analysis on regular gels, in addition the peptides do not bind the PVDF membrane. There is no MBP in the GFP fusions so the antibodies against MBP that are in the Y941 and Y936 antisera are not a problem.

We analyzed both N- and C-terminal fusions, based on prior experience with the anti-distal ER uORF peptide antiserum Y747 (see above in this task). Y941 clearly interacted with material of a size appropriate for a GFP fusion, but only when linked to the C-terminus of GFP. Y941 did not interact with protein where the peptide ORF was linked to the N-terminus of GFP.

Y941 rabbit antisera (bleed 2) worked first time in Western immunoblots of transfected HeLa cells, without any additional work up (Fig. 8). Note that we have expended considerable effort in

detecting the distal-peptide GFP fusion with antisera Y747, while, in contrast, Y941 is as easy to work with as commercial antisera against GFP, giving strong signal and low backgrounds (both to membrane and other HeLa proteins). These are excellent characteristics. Y941 gave a specific signal with the ER uORF prox-peptide fused to the C-terminus of GFP. Its gave no signal with cells transfected with the parent pEGFP construct. It also was conformation sensitive as seen for Y747 (against distal -GFP fusions) as it did not detect the prox-peptide fused N-terminal to GFP.

None of the other rabbit antisera against the ER uORF prox-peptide worked in the western blot format (Fig. 8). This is currently something of a paradox as Y935 and Y925 gave a strong signal in the BiaCore. We suspect we have reagents against at least two specific conformations/epitopes in the prox-peptide. Our future work is directed to testing application in an immuno-histochemical format which may or may not exhibit similar discrepancies.

We are developing model systems for testing antisera in an immuno-histochemical format. These test systems will ensure that we are detecting specific staining of target peptide when we test tissue sections. One can see in blots of Fig. 8 and elsewhere that sera may have interactions with proteins beyond the desired target. Our initial approach will be to use cells transfected with test constructs. Some of these are described in Fig. 9. The strategy is to have both peptide expression and GFP expression from one transcript. The detection of expressed GFP downstream in transcripts will give the confidence that the peptides are expressed. This system was tested in Fig. 10 using Western blots and probing for the expressed GFP with GFP antisera.

Tissue resources

Working with the cooperative human tissue network (CHTN) we have been fortunate to obtain a battery of breast tumor samples, mainly in fixed blocks. The paraffin blocks will be available to provide sections to analyze by immuno-histochemistry if adequate antisera are/have been developed. There is also the option of preparing RNA for direct analysis. This is an option if we cannot generate acceptable antisera and will be considered in the final phase of the project however; it will consume more material than immuno-histochemical analysis in blocks.

There is a summary of the samples in Table 2. A large fraction of the samples have matched non-tumor samples from the same patients. A wide range of ages are available. There are limited pathologist write ups on the samples but we would likely need to get the specific samples read for tumor stage/type as pathology provided is relates to the patient overall, not these specific blocks .
In addition we obtained a number of normal frozen samples, principally from reduction-mammoplasty subjects, and frozen tumor samples. Some of the frozen samples have been used for RNA isolations (see Task 3 and our fall 2002 Era of Hope poster). All sample acquisition was carried out within the protocols approved by the local IRB and by BCRP.

Task 2 Monoclonal reagents against Upstream peptides of the alternate Estrogen Receptor transcripts

Year 1:
    Initiate generation of monoclonals: schedule fusions
    Expand clones test supernatants
    Select preliminary group of positive clones for analysis based on immunoreactivity in ELISA &
BIAcore

Year 2:
    Characterize antisera in Western blots used phased program described in plan
    Apply antisera to immuno-histochemistry format on cells

Monclonal Antibodies

Our progress has been limited because of failure to generate usable monoclonal antibodies despite a good apparent immune response in the mice.

Immunogens used in an attempt to make monoclonal antisera were either raw full-length peptides (containing unique regionsand sequence shared by the alternate transcript ORFs, Table 1) or the maltose binding Protein (MBP) fusions in which the peptides were generated as recombinant C-terminal extensions of MBP (see cartoon, panel D in Fig. 15) grown in E. *coli*. Preparation of the recombinant ER-ORF peptide-MBP fusions was described in the prior report. Note that peptides included the shared region (Table 1) of five residues as we previously found that a rabbit polyclonal antiserum raised against the ER proximal promoter uORF peptide lacking this region would only react with the shortened peptide and would react with neither a full length prox-peptide containing the five shared C-terminal residues nor with a fusion protein containing the full length prox-peptide C-terminal to MBP.

Mice were initially immunized with peptides/proteins in complete Freund's adjuvant, and boosted twice at monthly intervals, the first with Freund's incomplete adjuvant, the second without.

A variety of tests were used to evaluate mouse sera and hybridomas however the BiaCore provides an effective means to screen for IgG positive clones and for activity against the immunizing peptides (Fig. 5, Table 3). The mice immunized with free peptide produced a substantial response. This is actually a significant point for the overall project (see work with rabbit polyclonals against the ER uORF prox peptide, Task 1). A sensorgram overlay is shown in Fig. 5. This is for serum from a mouse immunized with the free prox-peptide. The BiaCore chip has four sectors. One is blank to assess buffer effects (a problem in the BiaCore), a second measures IgG levels (antibody to IgG immobilized). The third and fourth carry the proximal and distal peptides attached via N-termini to the chip and allow detection of any interaction between serum antibodies and the peptides. A variety of parameters relating to interactions can be determined from sensorgrams, but at this point we are interested in crude assessment of interaction.

In all mice sera the signal to the IgG sector is strong (Table 3); this reflects general serum IgG. The signal with the prox-peptide is distinct while that with the distal peptide is weaker: Serum of mice immunized with the proximal peptide-related immunogens produced a better initial response than the distal sequences in terms of 'strength' and 'specificity'. For example (Fig. 5 & Table 3) the prox peptide antisera showed discrimination in the BiaCore for the prox peptide as immobilized test antigen over distal peptide. This was not seen with antisera from mice immunized with distal peptide (Table 3).

This could be partly due to generalized non-specific interactions and/or due to the fact that

peptides share five common C-terminal residues (Table 1). We are assuming that we can find and select mAbs that have a good discrimination between peptides. This is generally one of the advantages/opportunities in developing of mAbs. Note that data from last year indicated that excluding the shared regions from the immunogens was not a solution to the cross reaction problem: A polyclonal antiserum against the unique region of the prox peptide was strong when measured against the immunizing peptide in ELISAs but totally failed to react with the complete prox peptide. From accumulating data we suspect that the prox peptide has at least two antigenic epitopes (N-terminal and involving the shared C-terminal region respectively), while there may be only be a C-terminal epitope in the ER distal promoter uORF. The two peptides have distinct features. The overall pI of the prox peptide is much higher and it is more hydrophobic.

The spleens of mice immunized with free prox-peptide were selected for preparation of fusions. Hybridomas were produced using pre-tested reagent packages from StemCell Technologies. Clones were expanded in culture or in SCID mice for ascites. Culture supernatants were screened for IgG and reaction against the ER uORF peptides on the BiaCore instrument .

A population of clones was successfully obtained but the culture supernatants of positives were not as strongly reactive as the serum in the BiaCore (e.g. see Fig.6 for a clone that was subsequently used to generate ascites, compared to Fig. 5 for the serum of a mouse used for the fusions). In addition these supernatants were *not* positive in test Western blots against the MBP fusion. This is assumed to be a titer problem though it could be a conformation problem (see Task 1 on current analysis of polyclonal sera against prox-peptide). To obtain a higher titer we grew several clones as ascites. The ascites were even less active in the BiaCore and failed in western blots, except in a first experiment where we now assume something was mislabeled. We regrew the ascites of two clones and tested with and without freezing but the material was inactive. This is a setback for the project, tough we do now have several distinct and potentially effective rabbit polyclonals against the prox-peptide (see Task 1). We may go to external vendors to try again to make mAbs against the proximal ER-uORF peptide. This is dependent on how well the current rabbit polyclonals score in assessment as usable reagents.

We are requesting a our immunology core to schedule the preparation of monoclonal antibodies against the distal ER uORF peptide. From accumulating data we suspect that the prox peptide has at least two antigenic epitopes (N-terminal and involving the shared C-terminal region respectively), while there may be only be a C-terminal epitope in the distal ORF. The sera of mice immunized with distal peptide showed poor discrimination for distal peptide over the proximal peptide. However, one rabbit, Y747, did make an antisera specific to the distal peptide (see this and prior annual report). In making the request we are assuming that some clones will similarly make IgG that discriminates.

It is optimal to have distal and proximal ER-uORF immune reagents raised in different species and the significance of having the mouse mAbs against the ER distal promoter uORF peptide has increased now that the rabbit antisera against the prox peptide appear strong.

This has been a frustrating part of the project, however a positive point is that we noted the complete free prox pep and the prox-peptide MBP recombinant fusion produced a good immune responses. This is in contrast to the results with a short prox-peptide (linked to carrier) which generated only rabbit sera that reacted with the short immunogen. We therefore attempted new immunizations of rabbits with similar immunogen and this is producing encouraging, though complex, data (see Task 1) .

Task 3: Comparative analysis of ER alternate promoter use and the expression of translation products
Year 1
Refine RT-PCR methodology for analysis of ER promoter use in small samples from sections
Year 2-3
Begin study of relative ER alternate promoter use by RT-PCR in conjunction with antisera for peptides and for ER itself: In cell models and in tumor blocks.

We have in place the methodology for analysis of promoter use. We still need to prove methodology in RNA from blocks (fixed tissue) though we have demonstrated application to limiting numbers of cells.

RNA analysis

Establishment of assays for Proximal versus distal promoter use was described in the year one report. This assay was used to assay with a number of tumor samples. The material was from tumors samples that were obtained frozen rather than fixed (also see Task 1) . Several additional estrogen responsive markers were also assayed together with 28S RNA as a measure of RNA content (see Table 4). This data (Table 4) was described in our Fall 2002 Era of Hope poster (abstract included). In summary the very limited tumor set predominately used The ER proximal promoter when ER was expressed. Note that we consider PCR based assays to be primarily a research tool and that the immune reagents would be a better clinical tool. However the next step in application of the PCR based RNA assay would be to apply it to RNA from fixed blocks as this archived material is more available than unfixed material.

One use of the PCR based RNA assay for ER promoter use, and for several related cellular parameters, that we have discussed with a potential collaborator is the application to breast ductal lavage aspirates in women at high risk of breast cancer (3). Here the samples would be unfixed cells. We have not done any actual clinical work yet as the lavage system is not place. Our collaborator indicates that there is a need for molecular markers to better define cells that morphologically are difficult to stage as 'hyperplasia' or as "DCIS". We were able to do analysis on limited cell samples using a model system. The model system was the Ishikawa cell line which uses both distal and proximal promoters to generate ER transcripts. We were able to isolate RNA from 3000 cells using conventional (Trizol, Invitrogen) methodology and to assay both shared regions and promoter specific regions of ER RNA transcripts.

Our RealTime PCR assay could detect ER transcript shared regions and promoter specific regions in aliquots of the RNA from 3000 Ishikawa cells (Fig. 11). The melting curves were appropriate indicating specificity for the assay (Fig. 12).

Key research accomplishments

*A new antisera has been obtained which has the potential detecting one of the peptides encoded in the uptream regions of alternate Estrogen receptor promoter transcripts.

*It is clear that antisera can be developed against the peptides from both of the major alternate estrogen receptor promoter transcripts, though it is still unknown as to if the natural peptides survive in breast tumor cells at levels sufficient for detection and routine assay.

* Data indcte that conformation and route of presentation affect immunogenicity of the uORF apeprides and their subsequent reactivity with antisera

Reportable outcomes
Dr Fasco attended the Era of Hope meeting in Fall 2002 and presented a poster as required by contractual arrangements.

Dr Pentecost visited with Dr K Arcaro at U Mass at Amherst and to presented a seminar mainly on the work funded in this project (Oct 29, 2002).

Dr Pentecost had a minor role in a study of ER -negative derivatives of MCF-7. That projected utilized some of the resources of this study and the ER-negative lines will be used in testing antisera.
'Phenotypic Changes in MCF-7 Cells During Prolonged Exposure to Tamoxifen', Fasco et al, accepted by Molecular and Cellular endocrinology.

Conclusions
Our program is running behind schedule, but a expenditures have similarly been reduced so that we can likely afford to request of fourth year as a no-cost extension in order to more fully address the project goals. A major limitation has been and will be that it takes at least four months to generate a polyclonal antiserum in rabbits, and even longer to generate monoclonals. Hence our cycles of improvement are slow. The development of the immune reagents has been more challenging than expected when the proposal was written. However, the point of the studies; correlations between breast cancer disease and promoter use seems as valid as when proposed. We can make antisera against the upstream peptides embedded in the ER transcripts and the possibility remains to use these 'biomarkers'. There remains the risk that these peptides do not survive in breast tumor cells or that levels do not correlate with levels of transcripts.

Appendices

Appendices include the figures and tables of this report, the abstract of a poster presented last fall at the ERA of Hope meeting and a copy of the accepted manuscript prepared by Dr Fasco.

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4. **Kozak M** 1999 Initiation of translation in prokaryotes and eukaryotes. Gene 234:187-208

Region	DNA Sequence and Potential Translational Product
Distal 5' uORF	MetGluHisPheTrpLysAspValLeuAspProAlaGly**TrpProAlaGlyPheTer** ..caagccc<u>ATG</u>GAACATTTCTGGAAAGACGTTCTTGATCCAGCAGGG**TGGCCCGCCGGTTTCTGA**gcc...
Proximal 5' uORF	MetArgCysValAlaSerAsnLeuGlyLeuCysSerPheSerArg**TrpProAlaGlyPheTer** ..gcgggac<u>ATG</u>CGCTGCGTCGCCTCTAACCTCGGGCTGTGCTCTTTTTCCAG**GTGGCCCGCCGGTTTCTGA**gcc.
Major ER ORF	MetThrMetThrLeuHisThrLysAlaSerGlyMetAlaLeuLeuHisGlnIle .... ccacggacc<u>ATG</u>ACCATGACCCTCCACACCAAAGCATCTGGGATGGCCCTACTGCATCAGATCCA...
Kozak Optimal Translational Start	Met ... gccgccRcc<u>ATG</u>G...

Table 1: Translational Start Regions of Estrogen Receptor Open Reading Frames

The DNA sequences and entire potential encoded peptides of alternate ER transcript upstream regions are shown together with the fragment of the main ER coding region that is included in ER-GFP fusion constructs. The DNA sequences 5' of the translational starts are shown and are included in constructs as they contribute to the relative strength of translational start sites. The five shared codons (from a common exon) of the proximal and distal transcripts are shown in **bold**. For comparison the optimal translational start defined by Kozak (4) is included; the critical elements of the ATG flanking sequences are the purine (R) at -3 and the guanine residue at +4.

Sample state	Sample type	Number
fixed block	tumor	4
fixed block	tumor + matched normal	39
frozen	tumor	8
frozen	Normal/ Reduction mammoplasty	6

Table 2: Breast derived tissue samples available to the project

Tumor and normal breast tissue samples were obtained from the human cooperative tissue network

#	Target	Immunogen	Signal from specific chip sector		
			IgG	Prox	Distal
P0M	ER uORF **prox** peptide	prox peptide-MBP fusion	2560	**910**	257
P1M	ER uORF **prox** peptide	prox peptide-MBP fusion	2298	**1103**	235
P2P	ER uORF **prox** peptide	synthetic prox peptide	2217	**1053**	92
P0P	ER uORF **prox** peptide	synthetic prox peptide	2531	**1018**	96
D0M	ER uORF **distal** peptide	distal peptide-MBP fusion	2474	882	**288**
D4M	ER uORF **distal** peptide	distal peptide-MBP fusion	2207	621	**322**
D0P	ER uORF **distal** peptide	synthetic distal peptide	2165	803	**77**
D3P	ER uORF **distal** peptide	synthetic distal peptide	1902	833	**91**

Table 3: Generation of Antisera in mice as step for making Monoclonal Antibodies

Mice were immunized with either synthetic peptides to the uORFs of the Estrogen receptor proximal and distal promoter transcripts or with recombinant fusion proteins with the peptides C-terminal and in-frame with maltose binding protein (MBP).

The quality of immune response was assessed on the BiaCore instrument using a chip that has sectors that allow assessment, separately, of IgG content and immuno-reactivity towards distal and proximal peptides. The synthetic peptides are covalently coupled to the chip sectors and interaction with serum components is detected as a change in refractive index. This is presented as baseline normalized 'relative light units' (RLU).

All a mice had detectable IgG; an expected result. Serum from mice getting proximal peptide (as peptide or fusion) had a better response towards the proximal than mice getting the distal petide showed toward the distal peptide. In addition , the sera from mice immunized with proximal peptide or fusion showed much greater specificity toward the immunizing peptide, Note that the uORF peptides have five shared C-terminal residues (see Table 1).

Tumor ID	Type	Age	Clinical ER	Clinical PgR	ER %MCF-7	PgR %MCF-7	Comment	Tumor	28S	ER	RT-PCR PgR	Distal	Proximal	Dis+Prox /28S	ER/28S	PgR/28S	PgR/ER
21127	Mets to Lymph node	63	0%	0%	2.47%	275.00%		90%	0.0013	0.0025	0.47	0.000000	0.001600	1.26	1.93	373.76	193.74
23147	Malignant	47	0%	0%	0.94%	65.00%		80%	0.0003	0.0009	0.11	0.000000	0.000000	0.00	2.80	335.91	119.99
23153	Malignant		Negative	Negative				40%	0.0001	0.0000	0.03	0.000000	0.000000	0.00	4.19	195.16	47.3
23155	Malignant	47	Negative	Negative	0.74%	33.00%		90%	0.0009	0.0007	0.06	0.000000	0.000500	0.59	0.86	67.01	78.03
MCF-7	Cell Line		>90%	>90%	119.25%	275.00%		40%	0.0011	0.0990	0.1	0.000000	0.120000	112.68	92.96	93.90	1.01
23148	Malignant	47	0%	0%	2.83%	60.00%	20% Necrosis	80%	0.0001	0.0026	1.26	0.000000	0.000000	0.00	45.90	457.40	9.63
23149	Malignant	36	15%	20%	0.72%	4.45%	Weak ER Strong PgR	80%	0.0005	0.0007	0.01	0.000000	0.001100	0.00	1.58	16.99	10.74
23154	Malignant	60	26 fmol/mg	712 fmol/mg	2.17%	655.00%		80%	0.0007	0.0022	1.13	0.000000	0.001100	1.88	2.89	1517.55	525.85
23158	Malignant	53	90%	70%	2.88%	48.00%	Strong ER Weak PgR	100%	0.0004	0.0029	0.08	0.000000	0.000900	2.50	7.92	230.14	29.07
22392	Malignant	51	90%	10%	9.95%	42.50%		90%	0.0014	0.0099	0.07	0.001300	0.005500	5.04	7.30	54.34	7.45

Table 4: Data on breast cancer samples incorporated into ERA of Hope Poster, Fall 2003

28S

Tumor ID	Values		Average		% MCF-7
21127					119.25%
23147					31.36%
23153					12.21%
23155					79.81%
MCF-7					5.73%
23148					42.44%
23149					69.95%
23154					33.80%
23158					126.76%
22392					

ER

Tumor ID	Values		Average		% MCF-7
21127	0	0	0.002450		2.47%
23147	0	0	0.000935		0.94%
23153	0	0	0.000545		0.55%
23155	0	0	0.000730		0.74%
MCF-7	0.1	0.1	0.099000		
23148	0	0	0.002800		2.83%
23149	0	0	0.000715		0.72%
23154	0	0	0.002150		2.17%
23158	0	0	0.002850		2.88%
22392	0.01	0.01	0.009850		9.95%

PgR

Tumor ID	Values		Average	
21127	0.2	0.35	0.28	
23147	0.08	0.05	0.07	
23153	0.01	0.02	0.02	
23155	0.04	0.03	0.03	
MCF-7	0.1	0.1	0.1	
23149	0.19	0.13	0.16	
23154	0.01	0	0	
23158	0.66	0.65	0.66	
22392	0.04	0.05	0.05	

Distal Promoter

Tumor ID	Values		Average		No RT	amol/0.1 ugRNA	% ISH
21127	0	0	0		0	0	-22.69%
23147	0	0	0		0	0	-2.31%
23155	0	0	0		0	0	-2.78%
MCF-7	0.01	0.01	0.01		0	0	100.00%
ISH	0.01	0.01	0.01		0	-0	-72.69%
23149	0	0	0		0	0	-2.78%
23154	0	0	0		0	0	0.00%
23158	0	0	0		0	0	
22392	0	0	0		0	0	12.04%

Proximal Promoter

Tumor ID	Values		Average		No RT	amol/0.1 ugf	% ISH
21127	0.01	0	0		0	0.003400	0.001550	7.75%
23147	0	0	0		0	0.003100	-0.000750	-3.75%
23155	0	0	0		0	0.003000	0.000450	2.25%
MCF-7	0.12	0.12	0.12		0.118000	590.00%
ISH	0.02	0.02	0.02		0.020000	100.00%
23149	0	0	0		0.002200	-0.000350	-1.75%
23154	0	0	0		0.002700	0.001150	5.75%
23158	0	0	0		0.002500	0.000850	4.25%
22392	0.01	0.01	0.01		0.003000	0.005500	27.50%
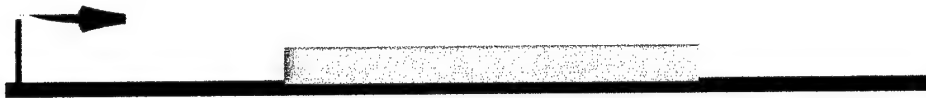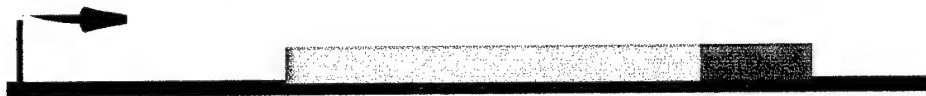
Fig. 1: Cartoon Illustrating some of the constructs designs utilized in studies

A. Our early cell reporter studies of the ER-uORF peptides utilized constructs based on Clontech's pEGFP-N1 which encodes a GFP protein expressed from a strong CMV promoter. This can be detected by its fluorescence in cells and as a protein in Western blots and immuno-histochemistry using anti-GFP antisera.

B. The majority of our cell studies utilized constructs in which the ER-uORF peptides were placed N-terminal to the GFP ORF in pEGFP-N1. The inserted fragments for the proximal and distal uORFs were included in separate constructs. The expression of the GFP fusion proteins can be detected as GFP fluorescence, GFP immuno-detectable protein in Western blots and potentially by suitable antisera against the uORF peptides

C. We created ER-uORF- GFP constructs in pEGFP-C1 after the failure of several antisera anti-ER uORF antisera to detect the relevant N-terminal GFP fusions. In these constructs the ER-uORF peptides are expressed as C-terminal extensions to GFP. The uORF peptides in the two types of GFP fusions may have differing conformations affecting detectability by antisera..

D. A tool in analyzing antisera and in making some antisera was a recombinant maltose binding protein E. *coli* expression system (see year one report) in which uORF peptides were expressed as a C-terminal extension of the easily purified MBP.

NS →
Distal C-term →
to GFP

Affinity purifed
Y747 anti-distal
peptide antiserum

Anti-GFP antiserum

1 2 3 4
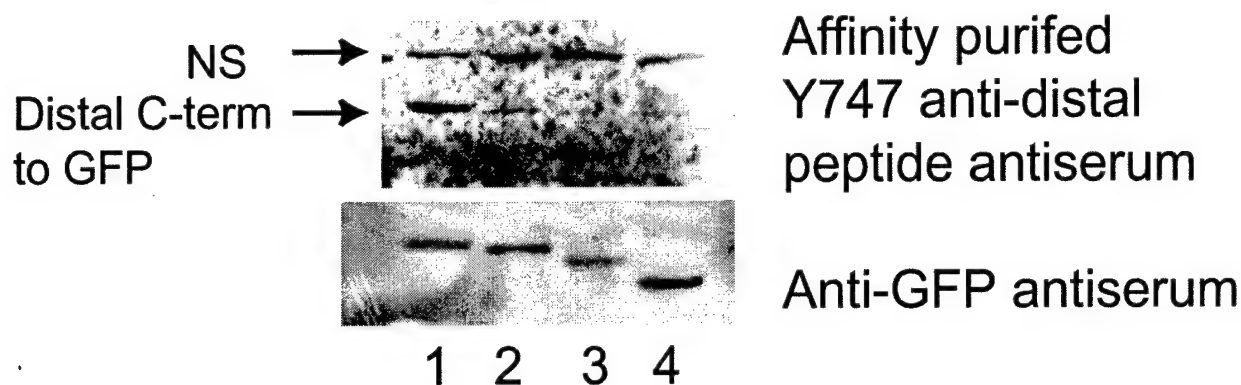
1. Distal peptide uORF fused C-terminal to GFP
2. Duplicate of transfect 1 (alternate construct)
3. Distal peptide uORF fused N-Terminal to GFP
4. GFP from parental construct pEGFPN1
(NS = non specific band)

Fig. 2: Y747 anti-serum detects ER uORF peptide C-terminal to GFP, but not N-terminal to GFP

Affinity purifed Y747 antiserum failed to detect a distal-uORF-GFP fusion in when the petide uORF was N-terminal to GFP, configuration we have routinely used instudies. The uORF placed C-terminal was detected. This was tested after consideration that the immunizing peptide had been tethered by its N-terminus to carrier for immunization and the detected form probably reflects effect of this conformation. Detection of GFPfusion by Y747 was challenging and at the limit of the reagent's usable range. This likely indicates that Y747 won't detect free peptide in ER positive cells. However the studies did provide a knowledge base thatwe applied to the new anti-prox peptide polyclonal antisera.
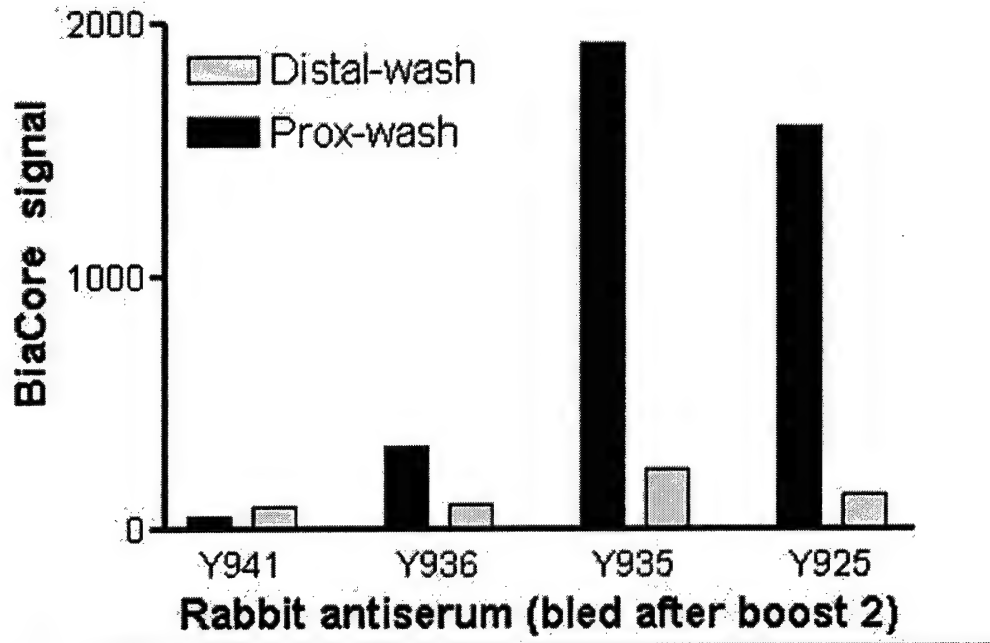
Fig. 3: BiaCore analysis of antisera against the ER uROF prox-peptide

Sera were drawn and analyzed 2 weeks after the second boost in incomplete Freund's adjuvant. Data for Y935 is susbet of data shown in figure 4 for test of all Y935 samples.
Y935 and Y925 show a strong signal in the BiaCore against the immobilized prox-petide with strong discrimination between the prox and distal sectors. These rabbits were immunized with the complete, but free and unconjugated, ER uORF prox peptide.
Y941 and Y936 antisera, that were raised against a recombinant protein with the prox-peptide fused to the C-terminus of Maltose Binding protein, show limited activity against the imobilized distal and prox peptide with poor discrimination for immunizing prox peptide sector.
Note that Y941 antiserum has minimal activity in the BiaCore assay yet is the only one of the four antisera that has good activity in Western blots against GFP-proxpeptide fusions.
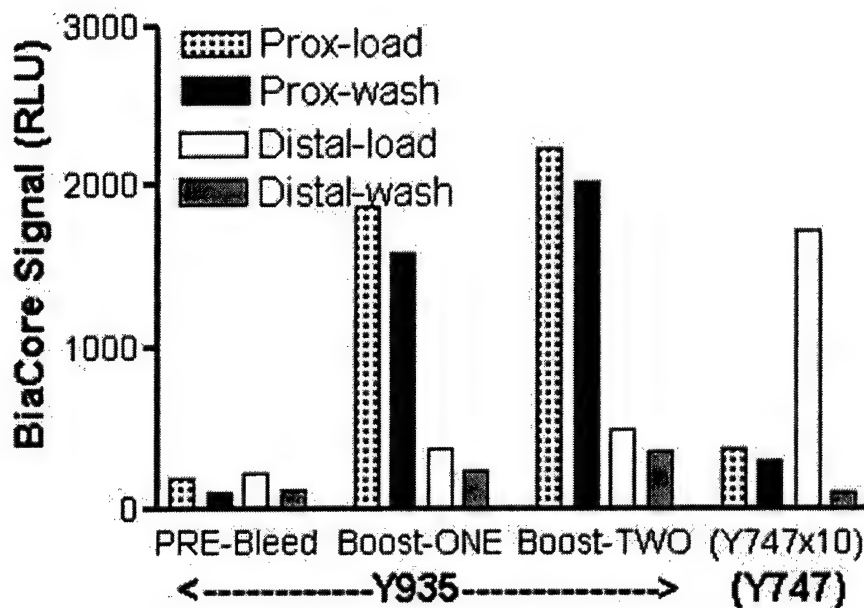
Fig. 4 : BiaCore analysis for Rabbit Y935 serum

BiaCore analysis indicates that serum from Y935 is developing a strong response against the immunizing prox-peptide. The serum has a high affinity component that does not disassociate during the pre-regeneration wash. That is in contrast with our earlier Y747 serum against distal peptide which is eliminated in the wash step. The Y935 serum interaction shows good discrimination for the proximal ER uORF peptide over the ER-uORF distal peptide with which it shares 5 C-terminal residues.

Note the current discrepancy that Y935 has excellent signal in this assay but not in western blots. Studies are refocusing on interaction in immunohistochemisty; our long-term application.
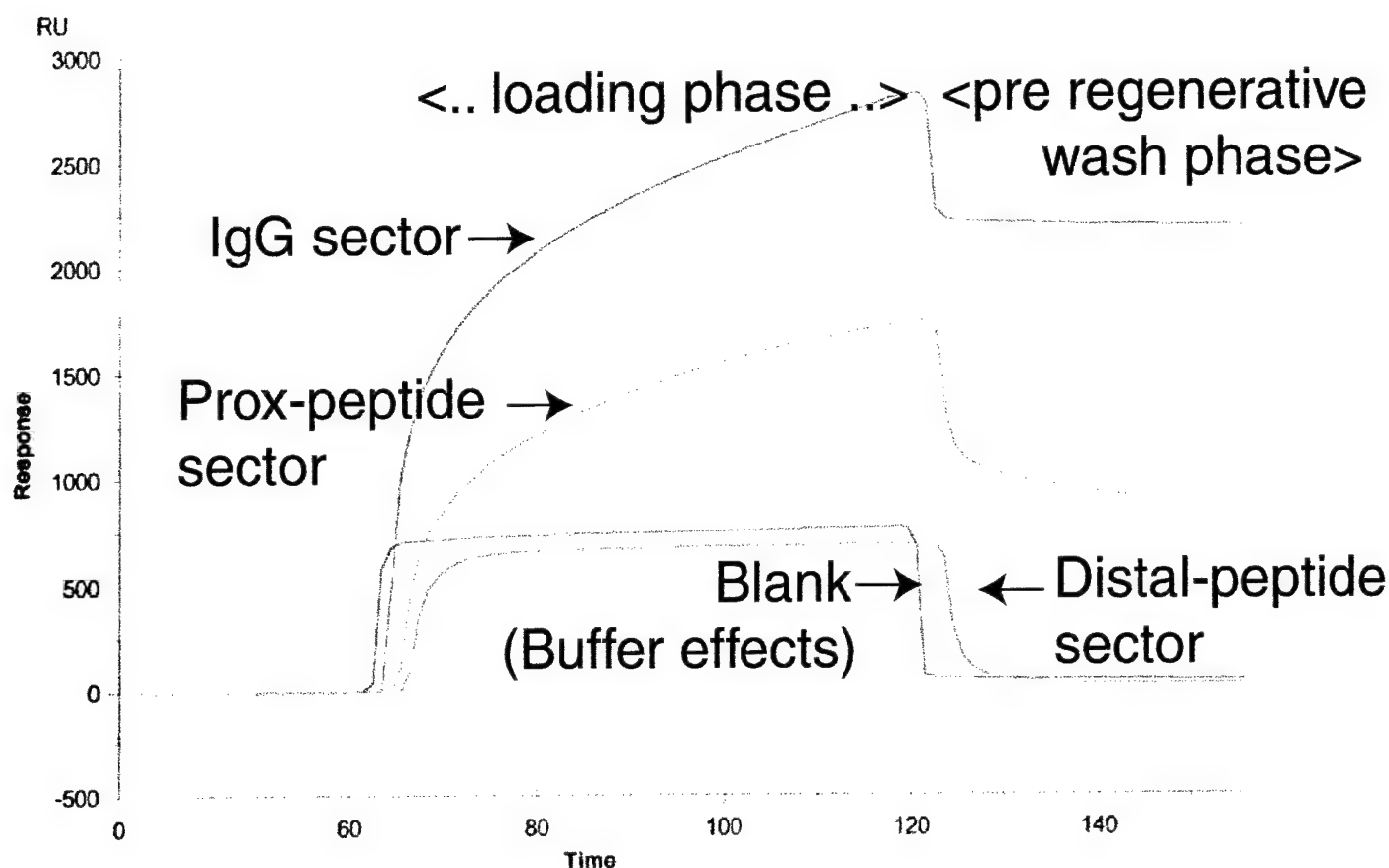
Fig. 5: BiaCore analysis of anti-prox peptide mouse serum prior to use of cells for mAb generation

Serum from one mouse used in fusions to generate mAbs. Serum was loaded onto to the chip and specific binding seen with sectors coated with IgG and with the Prox-peptide. This binding was partly resistant to elution in the pre-regenerative wash phase, indication high affinty interactions. Limited interaction was seen with the Distal-peptide sector and this was quickly lost in the pre-regenerative phase indicating weak interaction(ie there was good specifity for the prox-peptide).
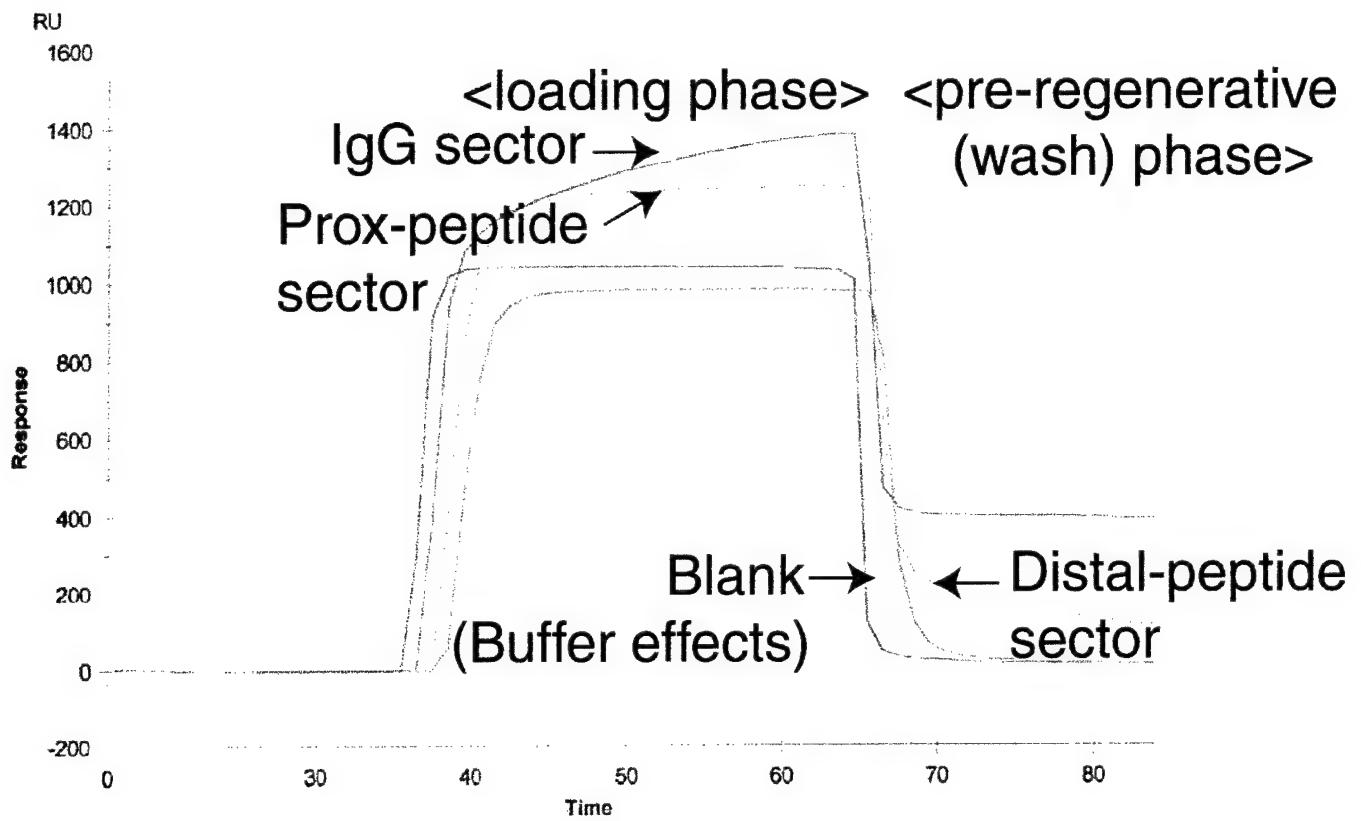
Fig. 6: BiaCore analysis of mAbs to prox-peptide

Supernatants of clones from fusions were analysed for expression of IgG and immunoreactiivity against the ER uOrF prox peptide. Activity against prox peptide was lower and lower affinity (washed out in pre-regenerative phase) compare to Fig. 5. Ascites grown from clones lost even this activity.
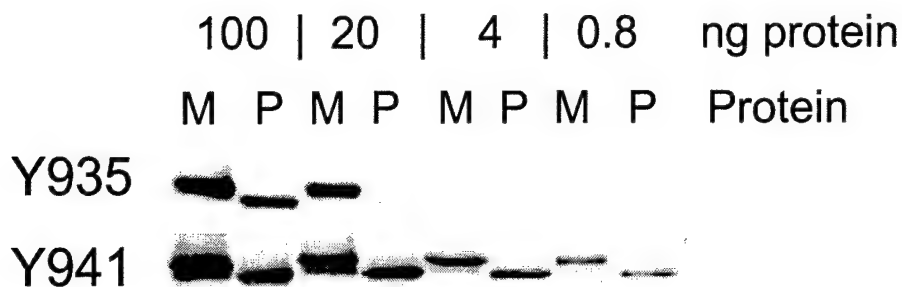
Fig. 7: Sensitivity and specificity of antisera against ER proximal promoter transcript uORF-peptide sequences

Western blots were prepared with serial dilutions of maltose binding protein (M) and a recombinant MBP-prox peptide fusion protein (P). These were probed with antisera raised against the free prox-peptide (Y935) and against the MBP prox-peptide fusion(Y941). The Y941 serum detected both the MBP and the fusion as the antiserum fusion probably contained specific antibodies against the MBP and the prox peptide (we attempted to deplete this anti-serum of the anti-MBP but were not succesful here).

At 20ng and 4ng of protein the Y935 antiserum could discriminate between MBP and the fusion protein, however there was a dramatic and unusual fall in signal between 20 ng and 4 ng for the fusion protein.

As seen in other figs there is a paradox in our data as Y941 does not give a good signal in the Biacore while Y935 does; but Y935 does not give good signal in any of the Western blot tests.
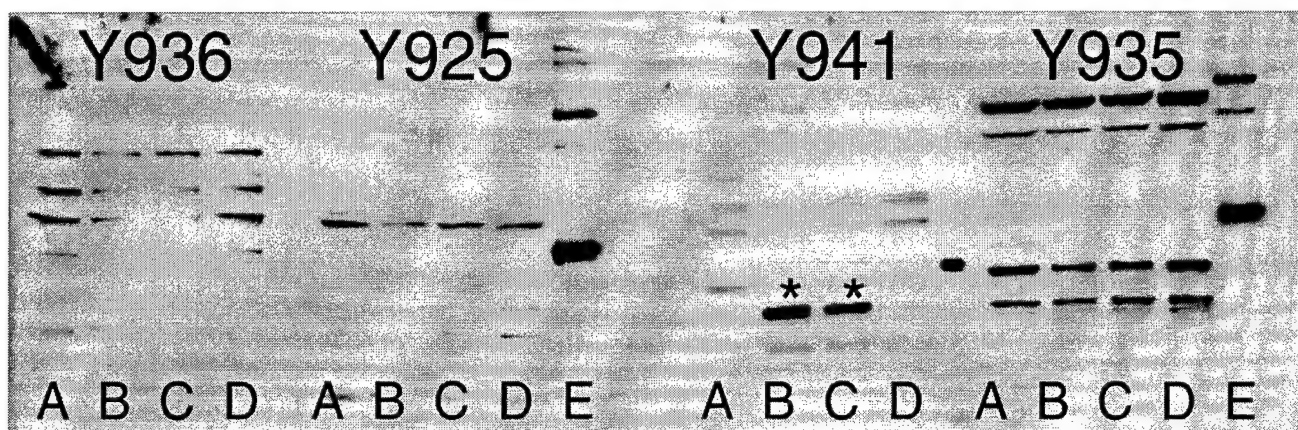
Fig. 8: Western blot assessment of Rabbit antisera against the ER uORF peptide

One of our tests for antisera is detection of GFP-fusion proteins in extracts from transiently transfected cells. This assay allows a measure of specificity for the ER distal/proximal uORF peptides versus general mammalian cell proteins. The fusions proteins run at about 20kDa in the gels and blots which makes them easy to analyze. Expression levels are likely high than for the actual peptide (in an ER expressing cell) as expression in the transfected cells is from a strong viral promoter, though only about 20% of cells get transfected. The actual uORF proteins are ~ca 2 kDA which is too small for analysis on regular gels, in addition the peptides do not bind the PVDF membrane.

Track A: GFP expression construct pEGFP-N1
Track B: GFP expression construct with prox peptide C-terminal to GFP in pEGFP-C1
Track C: Second GFP expression construct with prox peptide C-terminal to GFP in pEGFP-C1
Track D: GFP expression construct with prox peptide N-terminal to GFP in pEGFP-N1
Track E: 20 ng of the recombinant prox-MBP protein purified from E.coli (only included on blots for the rabbits immunized with un-conjugated peptides. The sera from rabbits immunized with the MBP fusion will have anti-MBP antibodies making analysis of MBP fusions in Western immunoblots irrelevant).
Also see construct descriptions in Fig *15 cartoon.

Y725:   Bleed 2 from rabbit receiving raw peptide as immunogen
Y735:   Bleed 2 from rabbit receiving raw peptide as immunogen
Y736:   Bleed 2 from rabbit receiving recombinant prox-peptide-MBP fusion as immunogen
Y741:   Bleed 2 from rabbit receiving recombinant prox-peptide-MBP fusion as immunogen
All sera were diluted 1:100 and detection was with the Pierce 'supersignal' chemiluminescent system.

Results: only the Y741 antiserum gives an appropriate (**) interaction with the prox-GFP protein in Western blots. The antiserum detects the Prox-peptide-GFP fusion protein in tracks B&C where the prox peptide sequence is C-terminal to the GFP ORF. However the antiserum is ineffective in detecting the prox peptide placed N-terminal to GFP in the transfected constructs. This data is in total contrast to the BicCore data.

E. Prior studies used GFP-fusion with ER-ORF peptides N-teminal

D. The GFP-fusion with ER-ORF peptides was unlinked by mutation

TGAATG
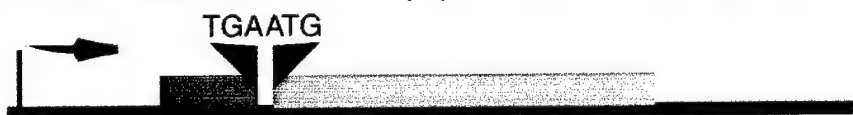
C. The strength of the uORF translational start was also increased

ATGTGAATG

B. Another existing construct set has the uORFs upstream of ER-GFP

A. GFP fusions with ER-ORF peptides C-teminal are available

Fig. 9: uORF peptide- GFP expression constructs for immuno histochemical studies

A number of constructs are available for use in testing the anti-peptide antisera in an immuno-histochemical format. Shown examples relate to the prox-peptide but similar are available for the distal peptide. The major principle is to be able to test for peptide expression while monitoring for GFP as a test that expression systems are working.

Some of the antisera can detect peptide fused to the C-terminus of GFP (A), however some could not in a Western blot format. An alternate construct available from prior studies is where the uORF is upstream of a fusion of GFP and the start region of ER (B), this is the natural format of the uORF. A problem for prox uORF constructs is that the translational start is weak, as determined form N-terminal GFP fusion constructs (E). Our anti-peptide antisera will not detect the N-terminal fusion to GFP (E), so as a test model we modified E by introducing a stop signal between the uORF and GFP (D). We had created constructs similar E where the prox-uORF translational start was mutated towards the optimal start motif (Table1) for higher expression. More recently we have modified these in a manner similar to D so that the more highly expressed uORF can now be expressed as a separate entity with GFP also expressed (C).

HEM45-GFP Reference →

Prox-ORFGFP →

GFP →

1 2 3 4 5
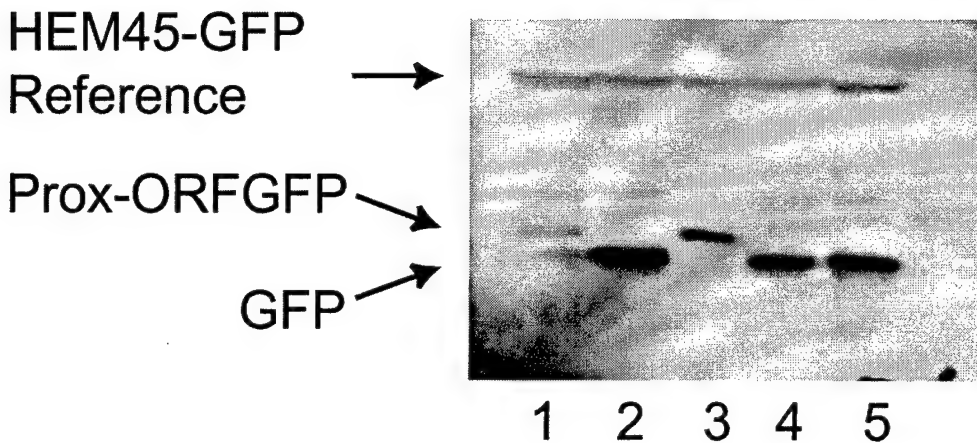
1. p2495-18: EGFPN-1 vector with prox uORF fused to GFP N-terminus (weak expression)
2. p3162-4: prox ORF and GFP from 1 separated by introduction of stop codon to prox uORF (GFP size decreased)
3. p2498-16: as 1 but uORF translational start region mutagenized towards optimum with 2 mutations to increase uORF expression.
4. p3164-10: as 3 but stop codon added like 2
5. pEGFPN1: parental GFP vector transfected to cells

Hela cells were transiently transfected with test GFP constructs along with HEM45-GFP wihch provides a transfection control. Blots of cell extracts were probed with anti-sera to GFP.

Fig. 10: Resources for prox-peptide analysis by immunohistochemistry with anti-peptide antisera

Recent data with new antisera against prox-peptide indicate that the ability of antisera to detect the peptide may be affected by the linking of peptide to 'carrier' we therefore need to move to test systems where the petide is not fused to carrier. These model systems are derived from existing fusion constructs (Fig. 9). We know the GFP fusions are expressed, we know the de-linked GFP is expressed. We can therefore have reasonable expectaton the prox-peptide is expressed.
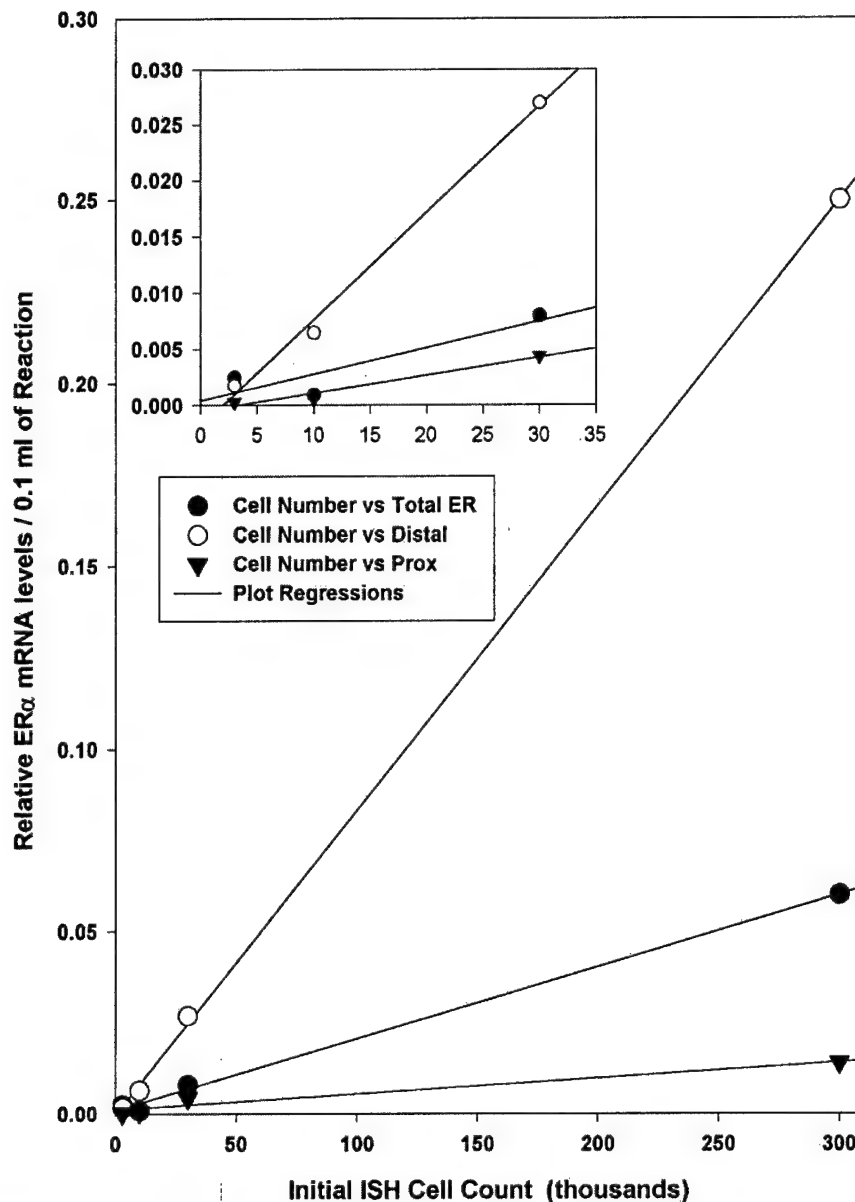
Fig. 11: Test of detection limits in ER RNA PCR
Ishikawa cells were used as a model to test the ability to analyze
RNA from limited cell numbers using RealTime PCR. Ishikawa
cells express ER RNAfrom both distal and proximal promoters
making them a useful model. RNA was isolated from cell samples
of 300K to 3000 cells. Aliquots of the RNA were analysed for total
ER mRNA and the promoter specific trancripts. Fair linearity was
achieved down to the smallest samples analyzed (aproximates
to RNA from 1000 cells). NOTE we only used one standard curve
to anlayze data. This ignores the different efficiencies of different
primer sets. This is why the distal yield appears higher than total.

Melting Curves of ER Distal and Proximal Promoter Transcripts
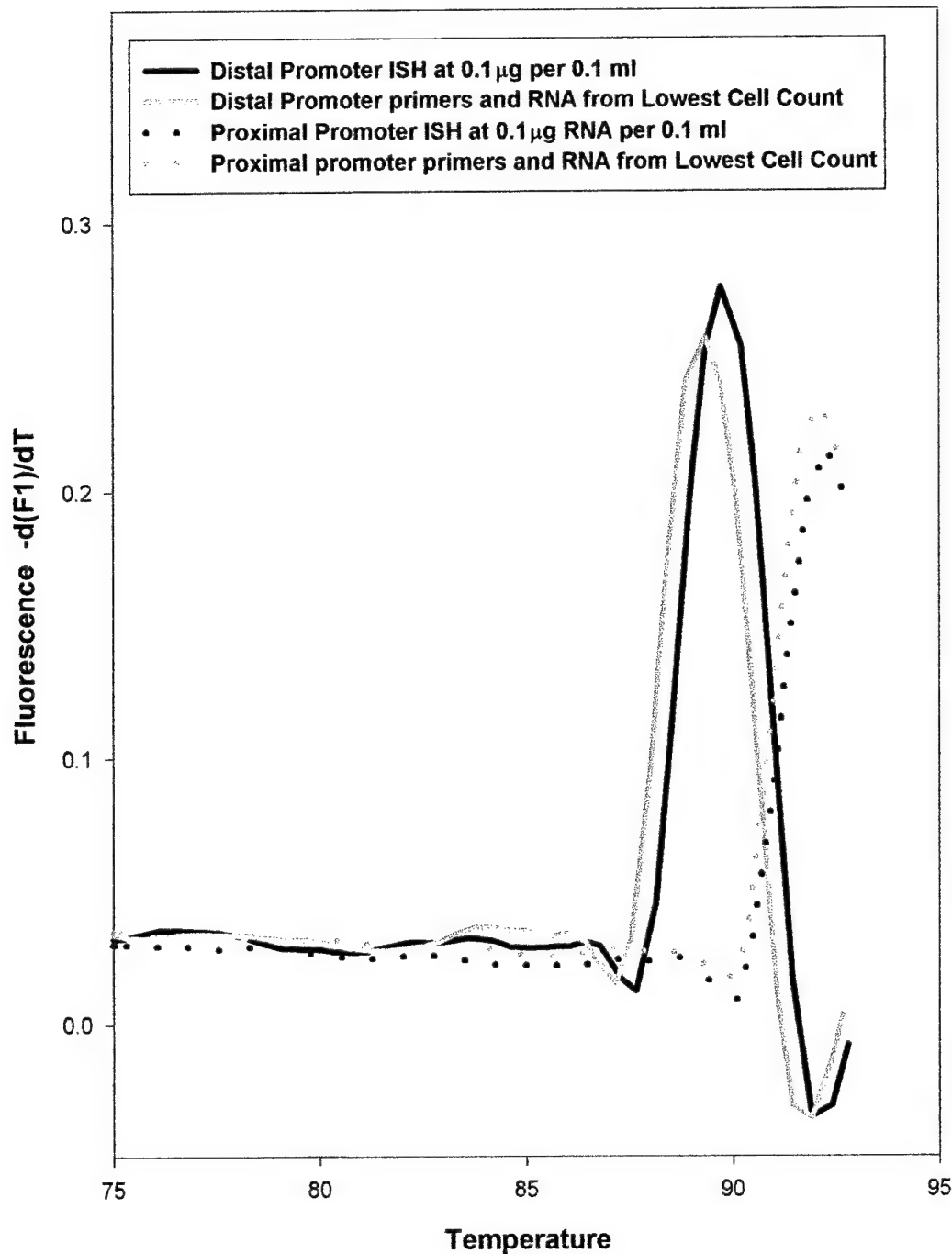
Fig. 12: Melting (denaturation) curves demonstrate the specificity of ER PCR amplification even when using only limited amounts of cells as starting material

ANALYSIS OF ALTERNATE ESTROGEN RECEPTOR PROMOTER USE USING NOVEL ASSAYS

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Estrogen Receptor alpha (ER) mRNA is primarily transcribed from two promoters. The proximal (prox) ER promoter transcribes a complete exon 1 whereas the distal promoter is 2 kb upstream in genomic DNA and splices into nt164 of the prox form. Identical ER protein is translated from both ER mRNAs because nt 164 is ~70 b upstream of the ER translational start. The two promoters may be under differing controls; their patterns of use may indicate how a breast tumor may progress and respond to drugs. We are developing assays to determine the promoter origin of ER transcripts and to test these hypotheses.

We developed a new one-step, real time RT-PCR method to quantify absolute levels of the mRNAs transcribed from ER distal and prox-promoters. Uterine Ishikawa cells contained 0.022 and 0.012 amol/µg total RNA of the prox and distal transcripts. The ratio of 1.8 is in good agreement with previously determined ratios of ~3. Consistent with our prior work, the breast tumor line MCF-7 had no detectable distal ER transcripts; the absolute ER prox-transcript concentration was 0.12 amol/µg total RNA. In contrast, two ER positive breast tumors had ER prox/distal mRNA concentrations of 0.0011/0.0003 and 0.0055/ 0.0013 amol/µg total RNA; ratios of 3.7 and 4.2, respectively. The data show that the very low levels of ER promoter transcripts can be quantitated by real time RT-PCR and suggest that the distal ER transcript is more prevalent in breast tumors than in breast tumor cell lines.

RNA-based assays are inherently challenging. We have therefore tried to develop protein based assays for alternate promoter use, despite the identity of ERs from the two promoters. The 5' region of prox-ER transcript contains a 20 amino acid (aa) residue open reading frame (uORF) besides the main ER ORF. The distal promoter transcript contains an 18 aa residue uORF, sharing five in-frame codons with the uORF in the ER prox transcript. Both uORFs terminate 50 bases 5' to the main ER translational start. From transfection studies we have evidence that the translational starts of the peptides are functional.

We propose that antisera can be made against the peptides and levels of the prox and distal peptides used as surrogate markers of ER promoter use. Rabbit polyclonal antisera were raised against the complete distal peptide, including residues shared with the prox uORF peptide. We obtained an antiserum that specifically interacts with a fusion protein of the distal peptide and maltose binding protein (MBP) in Western blots while interacting with neither MBP nor an MBP-prox-peptide fusion. This reagent will be further evaluated in test systems and tumor samples. A 15-residue prox-peptide containing unique sequences generated an antiserum with high ELISA reactivity to immunogen but the reagent failed to interact, in both ELISA and western blots, with prox-peptides containing the shared five C-terminal residues. Clearly the prox peptide has antigenic epitopes and further studies will involve use of modified immunogens and generation of mouse monoclonal antibodies.

Phenotypic Changes in MCF-7 Cells
During Prolonged Exposure to Tamoxifen[1]

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Running Title: Estrogen Receptor Expression

Footnotes

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[5] The abbreviations used are: DC5, Dulbecco's Minimum Essential Medium with supplements and 5% calf serum; E2, estradiol; RT-PCR, reverse transcription-polymerase chain reaction; ERα, estrogen receptor alpha wild type protein; ERα mRNA, estrogen receptor alpha wild type mRNA; ERβ, estrogen receptor protein beta; DEPC; diethylpyrocarbonate; ERΔn mRNA, estrogen receptor alpha mRNA missing exon n; ERΔnP, estrogen receptor alpha protein translated from ERΔn mRNA; EREs, estrogen response elements.

Key Words: human breast cancer; tamoxifen; estrogen receptor mRNA ; alternatively spliced estrogen receptor mRNAs; estrogen receptor protein expression and estrogen receptor transcriptional regulation

Abstract

MCF-7 breast tumor cells form multicellular nodules (foci) over a confluent monolayer in an estradiol (E2)-dependent, antiestrogen-sensitive reaction. A cell line cloned from MCF-7 that displays these phenotypes was probed to determine the effects of long term exposure to tamoxifen on the growth of foci , estrogen receptor alpha (ERα) status, and gene responsiveness to E2. In one of two experiments, a heterogeneous cell population emerged (TMX2) that over-expressed ERα mRNA (~20-fold) missing exon 3 (ERΔ3 mRNA) and its corresponding protein (ERΔ3P). On a per mRNA to protein basis, ERΔ3P and wild-type ERα were equivalently expressed. Return of the TMX2 population to medium without tamoxifen eventually selected for a population that expressed predominately wild-type ERα, whereas TMX2 clones over expressing ERΔ3 mRNA and ERΔ3P retained this phenotype in tamoxifen-free media. In both experiments, expression of all ERα mRNAs and proteins declined to barely detectable levels during six to twelve months exposure, concomitant with a progressive increase in the ability of the cells to form foci independently of E2 or tamoxifen presence. Selection for these various populations suggests that tamoxifen can induce and/or support certain cellular changes that lead to altered ERα expression, E2-independent cell growth and resistance to antiestrogens.

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Introduction

E2 [5] and its cognate receptors, ERα and ERβ, play pivotal roles throughout the life span of a female in such essential processes as cell differentiation and growth, organ development, reproduction, and maintenance of bone density and the cardiovascular system. E2 also serves as a mitogen that enhances the growth of cancer cells in tissues associated with reproduction – cancer of the breast being the most prevalent among Western women, as well as the most intensively studied. In normal human breast epithelium, ERβ may be the prevailing receptor, but during carcinogenesis ERα emerges as the overwhelmingly predominant form (1-3).

Basic tenets of the current paradigm for E2-regulated cell growth (4) are that circulating E2 enters the cell by passive transport, crosses the cytoplasm, and enters the nucleus, where it binds to ERα. Upon association with ligand, heat shock and other proteins bound to unliganded ERα are released. ERα then dimerizes and binds to EREs in the chromosomal DNA of responsive genes. Brown and co-workers (5) have recently demonstrated a stepwise assembly and cyclic grouping/ungrouping of E2-responsive transcriptional factors in MCF-7 cells. Their studies also suggested that the antiestrogenic compound, tamoxifen, caused recruitment of co-repressors instead of co-activators. In addition to binding to EREs, ERα-liganded dimers bind to proteins associated with AP-1 and SP-1 sites (6-9) and unliganded ERα may participate in transcription via ternary complex formation with cyclin D1 and steroid receptor co-activators (10,11). Given also the potential for ERα to form heterodimers with ERβ or other structurally different ERα proteins, and cross-talk between ERα with other protein synthetic pathways, unraveling the varied signaling and transcriptional mechanisms involved in the synthesis of

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proteins and growth factors that ultimately dictate E2-dependent growth in tumors is a formidable task.

Antiestrogens that bind to ERα and inhibit its growth function are classified into two groups. Type I antiestrogens possess both estrogenic and antiestrogenic activities in *in vitro* models; these are typically triphenylethylene compounds, represented by tamoxifen and its metabolites. Type II antiestrogens possess only antiestrogen activity and resemble E2 in structure. Type I and Type II antiestrogens have different effects on the two transcriptional activation function domains (AF-1 and AF-2) that reside in the N– and C-terminal regions of ERα (12), respectively, and only Type II antiestrogens consistently down-regulate ERα protein levels (13). E2 can also promote the down-regulation of ERα protein, but its effect is cell context-dependent (14). Tamoxifen has been the drug most commonly used in the treatment of ERα positive breast cancers because it selectively inhibits breast tissue ERα and may have beneficial effects on other receptor-positive tissues, such as bone (4).

Breast tumors that arise following long term tamoxifen treatment are often resistant to its growth inhibitory effect. In a within-patient cohort of 72 women with breast cancer who were treated with tamoxifen, the following observations were made: a) in most cases tamoxifen caused a reduction in tumor ERα concentration; b) most of the initially ERα positive tumors that acquired resistance to tamoxifen during treatment maintained ERα expression; and c) *de novo* resistant tumors tended to be ERα negative (15). Several possible mechanisms for tamoxifen resistance in ERα positive breast tumors have been explored including alterations in E2 metabolism, malfunction of the metabolic pathways essential for receptor function, and receptor

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mutation, particularly involving the role of splice variant ERα proteins, but a causal association has not been demonstrated (4).

ERα positive breast tumor cells deprived of E2 or exposed to antiestrogens for prolonged periods in culture eventually undergo phenotypic changes that allow them to proliferate under these normally growth-inhibitory conditions. Studies of cell lines displaying these phenotypes have provided insight into ERα function and mechanisms potentially responsible for the development of resistance to antiestrogens (16-18). Under the culture conditions used, MCF-7 cells grow to a confluent monolayer at a rate that is essentially independent of E2. Growth beyond this checkpoint occurs only very slowly in the absence of E2, but in its presence dome-like structures (foci) form over the monolayer in a dose-dependent reaction that can be quantified (19). The formation of foci is inhibited by Type I and Type II antiestrogens. In this study we used a line cloned (clone 33) from the parental MCF-7 cells that displays these phenotypes (19). Changes in E2-dependent postconfluent cell growth, ERα status, and gene responsiveness to E2 in these cells during prolonged exposure to tamoxifen were monitored.

Materials and Methods

Growth of MCF-7 Cells: The MCF-7 cell clone 33 (19) was maintained in T-75 culture flasks in DC5 without and with tamoxifen (10^{-6} M; Sigma, St. Louis, MO) added in DMSO (0.1% final concentration). Cells were suspended with trypsin (0.25%) and split 1 to 5 into fresh media every 3 days. Portions were assayed periodically for the formation of foci, and for ERα status.

MCF-7 Focus Assay: The MCF-7 focus assay, in which human breast cancer cells respond to E2 by producing multicellular nodules or foci on a confluent monolayer background, was conducted as previously described (19). Briefly, MCF-7 cells were suspended in DC5 after treatment with trypsin (0.25%), seeded into 24-well plastic tissue culture plates at a density of 1×10^5 cells/ml/well, and placed in a 37°C, humidified, CO_2 incubator. Cells were re-fed at 24 h and every 3-4 days thereafter with 2 ml of DC5 containing various concentrations of E2 and tamoxifen in DMSO (0.1% final concentration). After 14 days the cultures were fixed with formalin and stained with 1% Rhodamine B. The stained foci were counted using a New Brunswick Biotran II automated colony counter (Edison, NJ).

Whole-Cell Competitive ERα Binding Assay: The whole-cell ERα binding assay was similar to that previously reported (22). Briefly, MCF-7 cells suspended in DC5 were seeded into 24-well plates at a density of 5×10^5 cells/ml/well. Twenty-four h later, the seeding medium was changed to DC5 containing [2,4,6,7,16,17-^{3}H]E2 (1.0 nM) and varying concentrations of unlabeled E2, and incubated at 37°C for 3 h. The confluent cultures were then washed 3 times with PBS, 300 µl of ethanol was added to each well for 20 min to solubilize the bound [^{3}H]E2, and the radioactivity in 200 µl of the ethanol extract was determined. To ensure that E2 binding in the TMX and TMX2 cell lines and clones was specific, the amount of bound [^{3}H]E2 in the presence

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of increasing concentrations of unlabeled E2 was determined and compared with the replacement profile obtained with the parent MCF-7.

Cloning of TMX2 Cells: TMX2 cells in suspension were seeded into multiple 96-well plates at a concentration of 0.5 to 10 cells per 0.4 ml of DC5 per well to ensure that a significant number of wells contained single cells. After 16 h of incubation to allow attachment, the wells were assessed by microscopic examination, and those containing no cells or more than one cell were rejected. After one week of continued incubation, the remaining wells were assessed for single colony formation and these wells were scored as clones. As the clones expanded to 100 to 200 cells, they were trypsin-suspended, centrifuged at 1000 x g for 5 min, resuspended in 1 ml of DC5, and transferred to a 24-well plate well for further growth and analysis.

RNA extraction: RNA from T47D and the MCF-7 cell lines cultured in T75 flasks or 6-well plates was isolated with TRI reagent (Molecular Research Center, Cincinnati, OH) as described previously (39,40). RNA pellets were dissolved in DEPC water, and concentrations were calculated from the absorbance at 260 nm.

Real Time PCR : A Light Cycler[R] (Roche, Indianapolis, IN) was used with the Qiagen (Valencia, CA) One-Step Kit supplemented with Syber Green I (10,000x, Molecular Probes, Eugene OR). Master mixes were prepared at 4^0 C and in multiples of 50 µl containing: 33 µl water; 10 µl of 5X buffer (supplied); 2 µl of dNTP solution (supplied); 2 µl of enzyme mixture (supplied), 1 µl primer mixture (25 µM each); and 2 µl of Syber Green I (diluted 1/5000 with water). Aliquots (14.3 µl) were distributed into capillaries precooled to 4^0 C, and total RNA was added in 0.75 µl. Unknown samples contained 0.1 µg total RNA per µl for all genes assayed except 28S, which was 0.001 µg total RNA per µl. The capillaries were spun just prior to RT-

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PCR. RT was at 50^0 C for 30 min followed by a 15 min at 95^0 C heating step to inactivate the reverse transcriptases and activate the Taq polymerase. The number of amplification cycles was 45, and the cycling times for denaturation (95^0 C), annealing (60^0 C, except for PgR, which was 55^0 C), and extension (72^0 C) were, respectively, 15 sec, 15 sec, and 30 sec. The ERα primer set used for Real Time RT-PCR was: forward primer 5'-ATGATCAACTGGGCGAAGAG (exon 4; 1429-1448); reverse primer 5'-GATCTCCACCATGCCCTCTA(exon 6; 1613-1632) 204 bp (accession number NM_000125). ERβ primers were described by Vladusic and co-workers (41). The 28S primers were from Simpson and co-workers (42): forward primer 5'-TTGAAAATCCGGGGGAGAC; reverse primer 5'-ACATTGTTCCAACATGCCAG. The reverse PgR(AB) primer was from Fujimoto and co-workers (43): forward primer 5'-TGCGAGGTCACCAGCTCTTG; reverse primer 5'-TTTGCCCTTCAGAAGCGGAC (321 bp). The pS2 primers were: forward primer 5'-TTGTGGTTTTCCTGGTGTCA; reverse primer 5'-GCAGATCCCTGCAGAAGTGT 156 bp (accession number XM_009779) and the HEM45 (44) primers were: forward primer 5'-GAGCGCCTCCTACACAAGAG; reverse primer 5'-AAGCCGAAAGCCTCTAGTCC 188 bp (accession number U88964). Standard curves for ERα, pS2, HEM45, and 28S were generated from 10-fold dilutions of total RNA isolated from MCF-7 cells prior to the initiation of the experiment. The PgR standard curve was constructed from T47D total RNA. These curves were stored and used throughout this study to allow calculation of relative changes in target mRNAs. A standard RNA was always included with the unknown samples to compensate for run to run variation. Fluorescence at the end of each amplification cycle was acquired at 5^0 C below the start of the melting curve for each amplified target.

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ERα mRNA Splice Variant Compositions: The method was similar to that described previously (39,40), except that a one-step RT-PCR kit (Qiagen) was used. Master mixtures were prepared as described for Real Time RT-PCR, except that the Syber Green I was omitted and 50 μl reactions containing 2 μg of total RNA were run in a PE 9600 thermocycler (Applied Biosystems, Foster City, CA). Primer sets within exons 1 and 5, exons 4 and 8, and exons 4 and 6 was used to determine the proportion of various components in the ERα mRNA pool as reported previously (39). Amplification conditions were the same as used for Real Time RT-PCR, except that the extension at 72^0 C was for 1 min, and the number of amplification cycles was 35. Quantitation of the DNA products was done with a P/ACE 2200 capillary electrophoresis unit (Beckman Instruments, Fullerton, CA) equipped with an argon ion laser and a 47 cm x 0.75 μm μSil-DNA column (J&W Scientific, Fulsom, CA) as described (45). Primers spanning exon 3 were: 5'- GCTATGGAATCTGCCAAGGA (exon 2; 883-902) and 5'- AAGGCCAGGCTGTTCTTCTT (exon 4; 1264-1283) 401 bp (accession number NM_000125).

Nucleotide Sequencing: DNAs obtained by RT and amplification of TMX2 mRNA with the exon 1 to 5 primer set were separated on a 2% agarose gel and the ethidium bromide staining band at approximately 780 bp was isolated using the QIAquick kit (Qiagen) and protocol. A 1 μl portion was re-amplified in a 50 μl reaction using the exon 1 to 5 primer set and the components supplied in a HotStartTaq™ kit (Qiagen). The final magnesium concentration was 2.5 mM, and the concentration of each dNTP was 10 mM. The amplification conditions were as described above. The variant DNA was partially purified using a Qiaquick column (Qiagen) and cloned with a TA Cloning Kit (Clontech, Palo Alto, CA). Nucleotide sequencing of the insert-

positive clones was done at the Wadsworth Center Molecular Genetics Core Facility, using a PE-Biosystems ABI PRISM 377XL automated DNA sequencer and universal M13 primers.

Western Immunoblotting: Cells from the various lines were dissolved in SDS sample buffer without dye and 2-mercaptoethanol as described previously (20). Protein concentrations were calculated with a BCA kit (Pierce, Rockford, IL). Protein separation was done on 10% Bis-Tris gels under reducing conditions in MOPS-SDS running buffer, according to the manufacturer's protocol (NuPage, Invitrogen, Carlsbad, CA). Separated proteins were transferred to an Immobilon-P membrane (Owl Separation Systems, Woburn, MA) using the NuPage apparatus and protocol. Other conditions were as described (20). ERα specific antibodies were NCL-ER-6F11 (Nova Castra Laboratories, Newcastle, UK) and HC-20, H-184 and Ser118 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), used at a 1/100 and 1/500, 1/200 and 1/100 dilution, respectively, in blocking buffer. The ERβ specific antibody was N-19 (Santa Cruz Biotechnology) used at a 1/250 dilution in blocking buffer. Species- and Cruz molecular weight-compatible horseradish-peroxidase labeled secondary antibodies were from Santa Cruz Biotechnology Inc.

ERE-Luc Reporter: Estrogen receptor action was assessed using a firefly luciferase reporter system. The reporter construct (pERE-Luc) utilized a functional ERE as described (46), but it was placed upstream of the promoter-reporter cassette of pGL2-promoter. All DNA was prepared with the Qiagen maxi prep system. Cells were transfected in 24-well cluster plates using Lipofectamine 2000 (Invitrogen Life Technologies, Baltimore MD). Cell extracts were prepared with passive lysis buffer and assayed with the dual-luciferase assay (Promega, Madison WI). Fire fly luciferase activity was normalized to the Renilla luciferase activity from co-

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transfected pRL-CMV. pERE-Luc (180 ng) was combined with 20 ng pRL-CMV (Promega, Madison WI) and 300 ng pBluescript (Stratagene, La Jolla, CA) as carrier, to give 500 ng DNA for each well. This DNA was combined with 2 μl lipofectamine in 100 μl DMEM (phenol red-free). After 45 min this was added to cells plated the previous day at 125,000 cells per well. The cells were in 0.5 ml phenol red-free DMEM with stripped, de-lipidated serum (Sigma). The liposomes and media were aspirated after overnight (17 h) incubation and replaced with 2 ml DMEM (phenol red-free) containing serum replacement (Sigma), and the indicated level of E2 added with ethanol (0.1% final concentration). Cells were harvested after 47 h and the lysates were assayed in a luminometer (Berthold instruments). Corrections were applied to data for background autofluorescence. Media and liposomes were made up as master mixes that were subsequently aliquoted to multiple wells to minimize variability.

Results

MCF-7 clone 33, previously selected for its dependence on E2 for formation of foci (19), was continuously exposed to tamoxifen (10^{-6} M) in DC5, a highly E2-deficient media (<10^{-11} M). Clone 33 was also maintained in DC5 without tamoxifen throughout as a control population. Cell populations were assayed periodically for ERα status (splice variant composition and quantity) and changes in the patterns of postconfluent focal growth. When changes were observed, a portion was frozen for future use. Other portions were transferred to and maintained in media with or without tamoxifen. Two essentially identical experiments were conducted that started approximately one year apart. A box chart depicting the names of the cell populations used in this study, the approximate times when they were isolated, and under what culture conditions is presented in Fig. 1. Also in Fig. 1 are photographs illustrating the ability of the focus assay to detect postconfluent growth differences among different cell populations, and populations treated with E2 and antiestrogens. Panels A, B, and C show the E2-responsive parental MCF-7 cells cultured in, respectively: DC5 (E2-deficient environment); DC5 plus 10^{-10} M E2; and DC5 plus 10^{-10} M E2 plus 10^{-6} M raloxifene. Panels D, E, and F show the ERα negative TMX cells under the same culture conditions, except that in Panel F no E2 was added, and the antiestrogen was 10^{-6} M tamoxifen. The light gray areas are foci overgrowing the much darker appearing cells of the monolayer.

In the first of the two experiments, little change in ERα status was observed during the initial six months of culture in the presence of tamoxifen; a slight down-regulation of the ERα protein level did occur without any detectable change in the ERα mRNA splice variant pattern or the level of ERα mRNA. Subsequently, however, spontaneous (E2-independent)

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formation of foci began to increase. Concomitant with this phenotypic change was a progressive decrease in the expression levels of ERα and splice variant mRNAs and ERα. After approximately another six months, levels of ERα mRNA were just above the threshold for detection by RT-PCR, and ERα protein was undetectable in our Western immunoblot assay (Fig. 2). This ERα negative population (TMX), following transfer to media with tamoxifen (TMX(+)) or without tamoxifen (TMX(-)) more than one year ago, has not recovered any ERα mRNA or ERα expression nor has it undergone a reduction in the extent of E2-independent formation of foci. Postconfluent growth of foci in TMX occurs in the absence of E2, is not enhanced by E2, and is not inhibited by tamoxifen (Fig. 1).

In contrast to the first experiment, a pronounced change in ERα protein expression occurred in the second experiment during the initial 6 months of culture in tamoxifen. As shown in Fig. 2, two ERα proteins with approximate molecular masses of 66 kD and 61 kD were recognized in TMX2 cells (see Fig. 1 box chart) by anti-ERα antibodies reactive against epitopes in the N-terminal (NCL-ER-6F11) and the extreme C-terminal regions (HC-20). Not shown are Western immunoblots demonstrating that ERα and the 61 kD protein were also recognized by antibody H-184 (a polyclonal antibody raised against amino acids 2-185 of human ERα) and by Ser118 (raised against phosphorylated serine 118 of ERα). Reaction of both proteins with the latter antibody was greatly enhanced by exposing the cells to E2 (10^{-9} M) for 2 h before isolation of the cellular proteins. Recognition of the 61 kD protein by all of these antibodies strongly suggests that the amino acids missing from the ERα sequence are internal and not at the N – and C-terminal regions.

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Electropherograms of the DNAs amplified from cDNAs of the parental MCF-7 and

TMX2 cells with a primer set spanning exons 1 to 5 are illustrated in the two upper graphs in

Fig. 3. The DNA product amplified from those ERα cDNAs containing the complete exon 1 to

5 sequence (894 bp) is the major peak that elutes last in both electropherograms. The other

major peak (777 bp) in the electropherogram of amplified TMX2 cDNA is missing only those

nucleotides of exon 3 as established by nucleotide sequencing of TA clones containing the over-

expressed DNA purified from an agarose gel. The variant portion of the ERα sequence obtained

with the exon 1 to exon 5 primer set was (3' end of exon 2) TATTCAA **(Δ exon 3)**

GGGATACGAA (5' end of exon 4).

Complete mRNA splicing profiles in the parental MCF-7, TMX, and TMX2 populations

are presented in the bar graph of Fig. 3. The ERα mRNA splicing pattern in TMX was

determined by RT-PCR before the ERα mRNA diminished below the detection threshold. As is

evident from Fig. 3, TMX retained a mRNA splicing pattern that was very similar to that of

parental MCF-7 with the predominant mRNA forms being wild type and that missing exon 7. In

striking contrast is the TMX2 ERα mRNA pool that contains essentially equivalent amounts of

ERα, ERΔ3 and ERΔ7 mRNAs. In TMX2 cells, the percent contribution of ERΔ3 mRNA

expression to the total ERα mRNA pool is approximately 20-fold higher than in the other cell

lines (Fig. 3). This increase in ERΔ3 mRNA expression is accompanied by a nearly

proportionate decrease in the expression of wild type ERα mRNA; the net effect on the ERα

mRNA pool is that the percents of ERΔ3mRNA, ERΔ7mRNA and wild type ERα mRNA are

nearly equivalent. Since expression of ERα protein and the 61 kD ERα protein in these cells

occurs to nearly the same extent (Fig. 2; TMX2(-) or TMX2(+) cell extracts), it follows that the two proteins are similarly expressed on a per-mRNA basis. This contrasts with ERΔ7P, whose expression is much less than that of ERα on an equivalent mRNA basis (20).

Twenty-eight clones were isolated from TMX2 just after it was split into media with or without tamoxifen. A few clones expressed only ERα and one did not express any detectable receptor. All of the others expressed both ERα and the 61 kD ERα protein in various proportions, with the majority expressing approximately equivalent amounts of the two. TMX2-4 was selected as representative of the heterogeneous TMX2 culture because it expressed approximately equal amounts of the two receptors. TMX2-11 was selected as a representative of 61 kD ERα protein over-expression, having a 61 kD ERα to ERα protein ratio of approximately 4:1. Figure 4 qualitatively depicts the relationship that exists between the expression ratio of the 61 kD ERα protein to ERα protein (upper panel) and the expression ratio of the amplified ERΔ3 cDNA to complete sequence cDNA (lower panel) using primers located in exons 2 and 4. The fluorescence intensity of the complete sequence DNA band is derived from all the variant and wild type cDNA transcripts except for those missing primer locations in exons 2 or 4, which are not amplified, and those having enough internal nucleotide variation to produce DNAs that separate on an agarose gel. In this case, the internal variation was deletion of the nucleotides that comprise exon 3. As is evident, a highly positive correlation exists between the amount of ERΔ3 mRNA present in the ERα mRNA pool and the level of expression of the 61 kD protein; consistent with the concept that the 61 kD protein is translated from ERΔ3 mRNA.

Both of the TMX2 clones were transferred to and maintained in media without tamoxifen. Neither one has changed its levels of ERα and ERΔ3 mRNA or pattern of protein

expression since its isolation and maintenance in media without tamoxifen over 6 months ago. In contrast, the heterogeneous TMX2 culture underwent significant change depending on whether it was cultured in the presence or absence of tamoxifen. In the absence of tamoxifen (TMX2(-)), a population of cells gradually emerged that expressed primarily ERα mRNA and protein: compare TMX2(-) with TMX2-4, whose ERα and ERΔ3P expression is representative of the heterogeneous TMX2 population, and with the parental MCF-7. In the continued presence of tamoxifen (TMX2(+)) all forms of ERα mRNA and protein eventually diminished to the extent that no forms of the latter were detectable by Western immunoblotting. As indicated from the amplified cDNA profile shown in the bottom panel of Fig. 4, few if any of the now ERα protein negative TMX2(+) cells over-express ERΔ3 mRNA as this splice variant is only a minor component relative to the complete exon 2 to 4 sequence obtained.

Other truncated ERα proteins were also detectable in the cell populations expressing ERα and ERΔ3P (Fig. 4). We demonstrated previously that the 52 KD ERα protein in MCF-7 cells was ERΔ7P (20). In both of the TMX2 clones, and particularly in TMX2-11, another anti-ERα positive band was also detectable whose molecular mass was approximately 45 kD. The amino acid sequence of this protein is not known, but it is probably linked to the exon 3 deletion since it is detectable only in the populations that over-express ERΔ3P.

A comparison of the E2-binding capability among the various populations is presented in Fig. 5. The lower panel in Fig. 5 illustrates the relative amounts of total (wild type plus splice variant) ERα mRNA among the cell populations as determined by Real-Time RT-PCR. The corresponding ERα and 61 kD protein expression profiles are those depicted in Fig. 4. As

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expected, TMX and TMX2(+) cells, which had lost most of their ERα mRNA and ERα expression, also did not bind detectable E2. Each of the other populations bound the hormone efficiently, with TMX2-11 binding the most. Unlabeled E2 effectively competed for binding to the ERα protein(s) in a concentration-dependent manner that was very similar for all of the populations. Given some variation inherent in all the assays, relative differences between the cell populations at maximum (^{3}H)E2 binding (10^{-12} M unlabeled E2) closely approximate the relative levels of total ERα and ERΔ3P expression shown in Fig. 4 and the relative levels of their mRNA expression measured by Real Time RT-PCR (Fig 5. lower panel). Since the 61 kD protein constitutes 50% and 80% of the total ERα expressed in TMX2-4 and TMX2-11 cells, respectively, E2 must bind to the 61 kD protein and with an affinity comparable to that of the ERα. If this were not the case, E2 binding in these lines would be much less than in the parental MCF-7, which express only ERα.

To probe whether ERα function was significantly impaired by ERΔ3P, E2 effects on the endogenous ERα-regulated genes PgR, pS2 and HEM45 and on the non-regulated gene, 28S ribosomal RNA, were determined in all of the populations by Real Time RT-PCR (Fig. 6). In the ERα positive cell lines MCF-7, TMX2(-), TMX2-4 and TMX2-11, all three E2-dependent genes were significantly up-regulated by E2 ($p \leq 0.01$ for all except HEM45 in TMX2(-) which was 0.015) as determined by paired students t-test. E2 did not significantly increase mRNA transcription of the 28S non responsive gene in the various populations (p range = 0.10 to 0.65). Among the ERα negative lines, TMX cells showed a significant E2-dependent down-regulation of pS2 and HEM45, and the TMX2(+) cells a significant E2-dependent down-regulation of pS2.

The actual differences were quite small, however, and given the fact that endogenous levels of pS2 are substantially reduced in the ERα negative lines, it is possible that these differences are only statistical. Irrespective, regulation was in the opposite direction of those genes known to up-regulated by E2. TMX2-4 and TMX2-11 were also transfected with a construct containing an ERE and luciferase reporter (ER-Luc), and their response to E2 compared with that of parental MCF-7 (Fig. 7). An E2 concentration of 10 pM elicited a maximal and highly significant (paired t-test $p \leq 0.05$) increase in luciferase activity in MCF-7 and in TMX2-4 and TMX2-11 cells.

The effect of E2 concentration on the formation of foci among the various populations is illustrated in the line graph of Fig. 7. The bar graph shows the effect of 10^{-10} M E2 with or without 10^{-6} M tamoxifen on the formation of foci. TMX and TMX2(+), which are nearly devoid of ERα mRNAs and proteins, readily formed foci in the absence of E2 and independently of the presence of exogenous E2 at any concentration. Tamoxifen had little, if any, effect on the postconfluent cell growth of TMX or TMX2(+). The extents of formation of foci by the parental MCF-7 and TMX2(-) lines, which express, respectively, exclusively ERα and predominately ERα with some ERΔ3P, were similar with respect to E2-dependence and inhibition by tamoxifen. Much different, however, were the postconfluent growth patterns in the TMX2-4 and TMX2-11 clones. TMX2-11, and particularly TMX2-4, required more E2 for the formation of foci than did the parent MCF-7. Tamoxifen was an effective inhibitor of the formation of foci in TMX2-11, and probably TMX2-4 as well, although the data for this clone are equivocal because the focal density with or without E2 was very low.

ERβ mRNA expression in the parental MCF-7 cells and all the other populations was similar and very low, being just above the detection threshold of detection in our Real-Time RT-

PCR assay. No ERβ protein was detected by Western immunoblotting in any of the cell lines

(data not shown).

Discussion

Carcinogenesis involves the alteration of many genes, eventually resulting in unregulated growth and aggressive behavior of cells. Cancers arising in reproductive tissue are often dependent upon hormones, such as E2, for sustained growth and, while much has been learned regarding hormone-dependent cell growth, much yet remains unexplained. A major contributor to our lack of knowledge in this area is a shortage of adequate *in vitro* models. In the media used, MCF-7 cells readily grow to confluence in an essentially E2-independent manner, but continue to grow beyond this checkpoint point only very slowly (21, 22). Addition of physiological levels of E2 produces discrete, three-dimensional nodules of cellular overgrowth, termed foci, over the monolayer 3 to 4 days postconfluence. Their unique cellular structure readily permits quantitation of the number of foci per unit area by differential staining, thus assaying a highly complex growth process. The formation of foci is also sensitive to many xenoestrogens and xenoantiestrogens, and to Type I and Type II antiestrogens, thereby making the postconfluent cell growth of MCF-7 cells *in vitro* is a useful model by which to probe physiologically relevant mechanisms of hormone-dependent growth and antiestrogen resistance (19, 23-25).

During prolonged exposure of the parental MCF-7 cells to tamoxifen, populations of cells emerged in which changes in the levels of ERα mRNA and ERα protein expression and in the growth of foci could be readily detected without the aid of cloning. In two separate, but essentially identical experiments, both similar and markedly disparate changes occurred, despite the fact that the same initial cell population was used for each experiment. The MCF-7 parental line used here was originally derived from a single cell several years ago. Throughout these

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experiments clone 33 was maintained in DC5 (a highly E2 deficient environment _____

of $<10^{-11}$M) as a control population. Although cells cultured in an E2 deficient environment for

prolonged periods can undergo phenotypic changes (26,27), no detectable changes in the overall

E2-dependency on the postconfluent growth of foci or on ERα mRNA and protein expression

occurred during the two to three year course of these experiments. Recent subcloning of the

parental MCF-7 cell line did demonstrate that a few of the clones exhibited abnormal E2

dependency on the growth of foci, but none exhibited the phenotypes that developed during

prolonged exposure to tamoxifen.

In the first of our two tamoxifen exposure experiments, little change occurred in the

composition of the ERα mRNA wild type and splice variant pool or in the expression of their

corresponding proteins during the first 6 months. By the end of 12 months, however, a

pronounced reduction in the expression levels of ERα mRNA and protein had occurred. In the

second experiment, cells maintained in media containing tamoxifen also eventually lost ERα

mRNA and ERα expression while acquiring the ability to form foci independently of the

presence of E2 or tamoxifen. These TMX and TMX2(+) ERα negative populations (Fig. 1) have

retained these phenotypes since their transfer to tamoxifen-free media many months ago. One

clone isolated from the heterogeneous TMX2 population also lacks detectable ERα proteins and

forms foci independently of the presence of E2 and tamoxifen (data not shown), adding support

to the observation that ERα loss associates with enhanced rates of postconfluent cell growth in

the MCF-7 model. Oesterreich and co-workers (28) also found that ERα negative MCF-7

clones (isolated from cultures deprived of E2) exhibited increased basal cell growth relative to

the parent MCF-7 population, and that E2 was not a growth stimulant. These hormone-

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independent and tamoxifen-insensitive growth characteristics in the ERα negative cells thus mimic the more uncontrolled and aggressive growth phenotypes associated with ERα negative breast tumors and may provide useful models to uncover novel cellular pathways associated with this phenotype.

During the first 6 months of the second of the two exposure experiments, a novel population (TMX2) emerged that over-expressed a 61 kD ERα protein and an ERα mRNA that was demonstrated by nucleotide sequencing to be missing exon 3. Several subsequent studies detailed in the Results section provided compelling evidence that the 61 kD ERα protein is missing the 39 internal amino acids encoded by exon 3. The two designations of 61 kD protein and ERΔ3P are therefore interchangeable throughout this text.

Many alternatively spliced ERα mRNA variants have been described (29-31), but only in a few instances have expression of their corresponding proteins been detected. ERΔ5P and ERΔ4P were detected in breast tumors and ovarian normal and tumor tissue extracts, respectively (32, 33). A 77 kD ERα expressed from ERα mRNA containing duplications in exons 6 and 7 is also expressed in an MCF-7 cell clone that grows optimally in the absence of E2 (27). ERΔ7P is expressed in the ERα positive breast tumor cell lines MCF-7, T47D, and ZR-75, and in some ERα-positive tissues and tumors (20). To our knowledge, this study records the first time that ERΔ3P has been detected as a naturally occurring variant. Particularly striking is that its amount in many of the clones isolated exceeds the amount of ERα.

The isolation of cell populations expressing various ERΔ3P/ERα ratios in a normally regulated cellular environment provides a unique opportunity to examine the effects of ERΔ3P

expression on a variety of E2-dependent processes. Of the identified ERα splice variants, ERΔ3P most resembles ERα, missing only 39 amino acids in the DNA binding domain. Because ERΔ3P efficiently binds E2, it possesses the potential to form liganded heterodimers with ERα and ERβ or homodimers that could possess dominant negative or dominant positive transcriptional activities or affect cross-talk pathways. Cells expressing exclusively ERΔ3P could also be used to probe transcriptional events of ERα that are not dependent on its DNA binding domain. Most importantly, steady-state ratios of ERΔ3P/ERΔ3 mRNA are equivalent to those of ERα demonstrating that this receptor form is not a target for a reduced rate of synthesis and/or enhanced degradation as occurs with ERΔ7P (20). ERΔ3 mRNA has been found in normal breast epithelium (34) and in a variety of ERα positive cell lines and breast tumors (29, 35). If the level of ERΔ3 mRNA expression is sufficient to support translation, it is probable that ERΔ3P is also expressed in these tissues and tumors.

Cell lines that over-express ERΔ3P also offer a means to evaluate how a naturally expressed ERα variant affects E2-dependent reactions compared to ERΔ3P expressed in cells from a transfected gene. In the two ERΔ3P expressing clones studied here, whose ERΔ3P to ERα ratios were approximately 1:1 and 4:1, E2 effectively up-regulated the endogenous E2-dependent genes PgR, pS2 and HEM45 and a luciferase reporter containing an ERE, demonstrating that ERΔ3P does not possess strong dominant negative activity against ERα. In contrast, ERΔ3P expressed from a transfected gene exhibited strong dominant negative activity against a similar ERE construct (36), and MCF-7 cells expressing ERΔ3P from a transfected gene exhibited reduced anchorage-independent growth as well as a 93% reduction in E2

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mediated pS2 up-regulation (34). A unique and not well understood aspect of E2-regulated cell growth is that some of the E2-dependent pathways are subject to "uncoupling", such as occurs in the ERα positive / PgR negative breast tumor phenotype. Based on the studies presented here, installation of ERα or any its variants by transfection does not ensure restoration of the actual E2-dependent receptor functions that normally occur within a cell. Indeed, in only one published study have ERα-transfected cells been isolated that exhibit an E2-dependent, positive growth response and this occurrence was from a MCF-7 cell clone that had lost its receptor during long-term E2 withdrawal (28). ERα-negative cells transfected with ERα are normally growth-inhibited by E2 (37, 38).

The cellular phenotypes responsible for the emergence of cells that over-express ERΔ3P in an environment deficient in E2 and rich in the agonist/antagonist tamoxifen are an enigma. Culture of the TMX2-4 and TMX2-11 clones in E2-deficient, tamoxifen-free media for a prolonged period has not altered their ERΔ3P/ERα expression demonstrating that ERΔ3P expression is not epigenetic, but due to a stable genomic modification. When the heterogeneous TMX2 population was transferred to the same medium, ERΔ3P over expressing cells essentially disappeared and a population emerged that expressed primarily wild-type ERα; suggesting that ERΔ3P over expressing cells somehow require tamoxifen for their survival in a mixed cell milieu. However, in the TMX2-4 and TMX-11 clones examined, postconfluent cell growth of foci was inhibited by tamoxifen and required much higher levels of E2 than the parent cells. Additionally, no obvious correlation exists between the ability of E2 to support postconfluent cell growth and the presence of ERΔ3P. TMX2-11 cells expresses nearly twice as much ERα

and ERΔ3P combined than do TMX2-4 cells, and ERΔ3P/ERα at a proportion approximately 1.5 times higher, yet the TMX2-4 cells are much less responsive to E2 with respect to the growth of foci. Preliminary studies with the other clones co-expressing ERΔ3P and ERα at various concentrations and proportions (data not shown) have similarly failed to indicate that a relationship exists between the E2-dependent growth of foci and the presence of ERΔ3P. A transcriptionally functional receptor does apparently exist in the TMX2-4 and TMX2-11 clones, however, because E2 up-regulated all the E2-dependent genes examined as efficiently as it does in the parent MCF-7 cells (Fig. 6). From a mechanistic perspective, therefore, it appears that factors other than, or in addition to, the expression of ERΔ3P might be involved in the responses observed and more in-depth studies regarding the role of ERΔ3P in E2-dependent transcription and the cellular pathways that participate in E2-regulated growth. Whether ERΔ3P over-expression also occurs in breast tumors, and particularly in the tumors of patients who have received tamoxifen for a prolonged period or whose ERα positive tumors have become tamoxifen resistant also awaits future study.

In summary, prolonged exposure to tamoxifen produced ERα protein expression and cell-survival and growth changes in our MCF-7 cell line that were detectable without the aid of cloning. ERΔ3P was identified as an over expressed protein in a heterogeneous cell population cultured in an E2-deficient medium containing tamoxifen. Return of this population to media without tamoxifen resulted in a marked reduction in ERΔ3P over expressing cells, but a similar reversion did not occur with ERΔ3P over expressing cells that were cloned from the TMX2 population. Cloned TMX2 cells naturally over expressing ERΔ3P were E2-responsive toward

three endogenous E2-dependent genes, and toward an ERE construct demonstrating that ERΔ3P

is a not a strong dominant negative inhibitor of ERα transcriptional activity as had been

suggested by transfection studies. Paradoxically, however, these clones require much higher

levels of E2 to attain focus formation compared to the parental MCF-7 population. Of the

populations examined in the tamoxifen exposure experiments, only those that had lost ERα

mRNA and protein expression were tamoxifen-resistant in the focus assay. These populations

also formed foci independently of E2.

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Figure 1. Box chart showing the names of the various MCF-7 populations used in this study, when they were isolated and under what culture conditions. Phase contrast microscopy of 10-day postconfluent cell growth in the ERα positive MCF-7 parental cells and the ERα negative TMX(-) cell population. Cells showing as dark are the confluent monolayer; light areas are the foci. Panel A, MCF-7 parental cells in DC5 and thus without E2; panel B, MCF-7 parental cells in DC5 plus 10^{-10}M E2; panel C, MCF-7 parental cells in DC5 plus 10^{-10}M E2 and 10^{-6}M LY-156758 (raloxifene); panel D, ERα negative TMX(-) cells without E2; panel E, TMX(-) cells plus 10^{-10}M E2; panel F, TMX(-) cells plus 10^{-6}M tamoxifen. For growth conditions see Materials and Methods.

Figure 2. Western immunoblots of various MCF-7 populations that developed during continuous exposure to tamoxifen (10^{-6}M). MCF-7 cells were continuously cultured in DC5 without tamoxifen. The NCL-ER-6F11 antibody recognizes the N-terminus of ERα and HC-20 recognizes amino acids in the extreme C-terminus. The ERα negative TMX cells that developed after approximately one year in tamoxifen presence were transferred to media without tamoxifen (TMX(-) cells) and with tamoxifen (TMX(+) cells) and assayed when they reached confluence. The TMX2 population at the end of 6 months in culture expressed high levels of ERα and a 61 kD ERα protein. It was also split into media without tamoxifen (TMX2(-) cells) and with tamoxifen (TMX2(+) cells) and assayed at confluence. Each lane contained 20 µg of total protein. Other conditions were as described under Materials and Methods.

Figure 3. ERα mRNA alternative mRNA splicing in parental MCF-7 and in TMX and TMX2.

Top: electropherograms of MCF-7 (left) and TMX2 (right) DNAs amplified from ERα cDNA

with the exon 1 to 5 primer set. The identity of the product missing exon 3 in the TMX2 cells

was established by nucleotide sequencing. Bottom: bar graph of complete alternative ERα

mRNA splicing patterns in MCF-7, TMX, and TMX2 constructed DNA fragments amplified

with the exon 1 to 5 and exon 4 to 8 primer sets. The splicing pattern in TMX was obtained

before the level of the ERα mRNA pool dropped below the analytical limit of the assay.

Conditions were as described under Materials and Methods.

Figure 4. Upper panel: Western immunoblot depicting receptor expression differences that

occurred when the TMX2 cells previously exposed to tamoxifen for six months (see Fig. 1 box

chart) were continued in culture in the presence (+) and absence(-) of tamoxifen (10^{-6} M) for an

additional six months. Note the differences in the expression profiles of these cells compared to

those in the TMX2(+) and TMX2(-) cells determined six months earlier (Fig. 2). The antibody

was NCL-ER-6F11, and the total protein per lane was 20 µg. TMX was from the original ERα

negative line and is included as an ERα negative reference. TMX-4 and TMX-11 are clones of

TMX2 (Fig. 1 box chart) that had been cultured in the absence of tamoxifen for approximately

the same length of time as TMX2(-) cells shown here. Bottom panel: RT-PCR of the RNAs

from these cells using the exon 2 to 4 primer set are included so that the amount of ERΔ3 mRNA

expression in the various samples can be compared to the amount of the 61 kD protein

expression (ERΔ3P). Experimental conditions were as described under Materials and Methods.

Figure 5. Upper panel: comparison of whole-cell E2 binding among the various MCF-7 cell

populations. Error bars are the SEM of four replicates. Lower panel: relative amounts of total ERα mRNA in the various MCF-7 cell populations excluding the contribution from alternatively spliced ERα mRNA variants missing exons 4 or 6, which comprise no more than 15% of the total ERα mRNA pool (see Fig. 3; -exon 4 plus forms contained in "other"). Values are the average of duplicate samples run as described under Materials and Methods for Real-Time RT-PCR of ERα mRNAs. Corresponding ERα protein expression is shown in the Western immunoblot in Fig. 4.

Figure 6. Comparison of E2 deprivation (DC5 media only – dark bars) versus 24 h E2 exposure (10^{-9} M – gray bars) on mRNA levels of the E2-dependent genes PgR, pS2, and HEM45 and the E2-independent gene 28S among the various cell populations. Values are the mean $\pm$ SD of two separate experiments containing duplicate samples. Significance was determined by paired t-test between the DC5 versus E2 exposed samples: $p \leq 0.05$ and ≤ 0.01 are represented by 1 and 2 stars respectively. Second experiment samples for 28S in the E2-treated 2-11 cells were lost, hence the absence of an error bar. Conditions for Real-Time RT-PCR were as described under Materials and Methods. ERα and ERΔ3P protein expression is that shown in the Western immunoblot of Fig. 4. Names designated with a 2- are abbreviations for TMX2. Also included in the figure is a comparison of the ability of E2 to activate a transfected construct containing an ERE and luciferase reporter (ER-Luc) in the parental MCF-7 cells (black bars) and the ERΔ3P-expressing clones TMX2-4 (light grey bars) and TMX2-11 (dark grey bars). Values are the SEM of triplicate assays. Statistical comparison by paired t-test (* = $p \leq 0.05$) is shown only for the three cell lines not exposed to E2 versus the same cells exposed to 10 pM E2 . Experimental

conditions for preparation of the construct and for the assay of luciferase activity were as described under Materials and Methods.

Figure 7.   Sensitivity of foci formation to the presence of E2 and tamoxifen among the various MCF-7 cell populations. Upper panel: comparison of postconfluent formation of foci by the various cell populations as a function of E2 concentration. ERα protein expression is that shown in the Western immunoblot of Fig. 4. Bottom panel: comparison of tamoxifen (10^{-6}M) inhibition of E2 (10^{-10}M) induction of postconfluent formation of foci among the various cell populations. Error bars are the SEM of four replicates. Experimental conditions for the focus assay were as described under Materials and Methods.

Figure 1
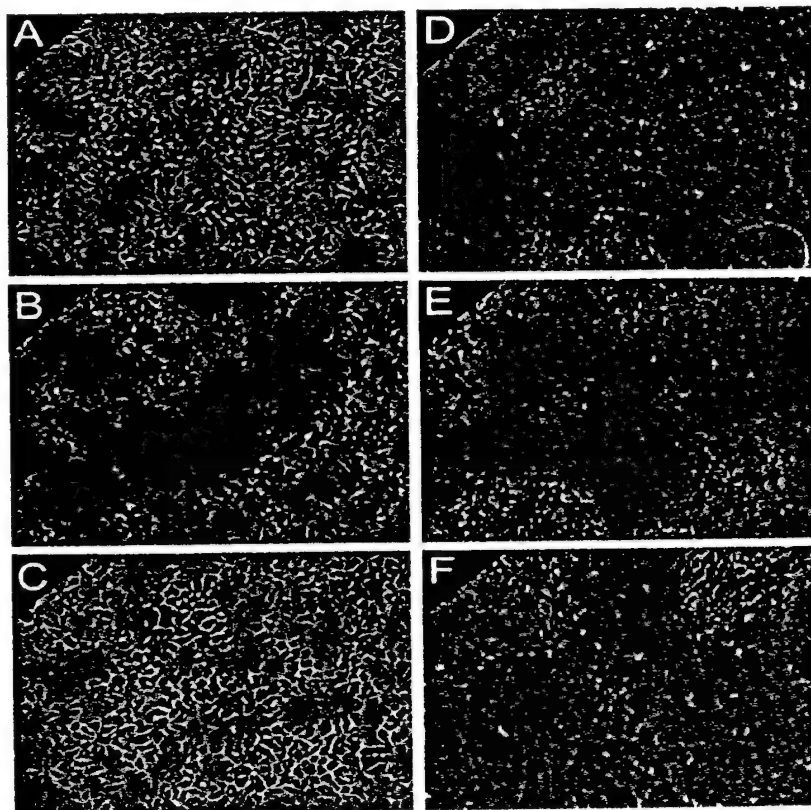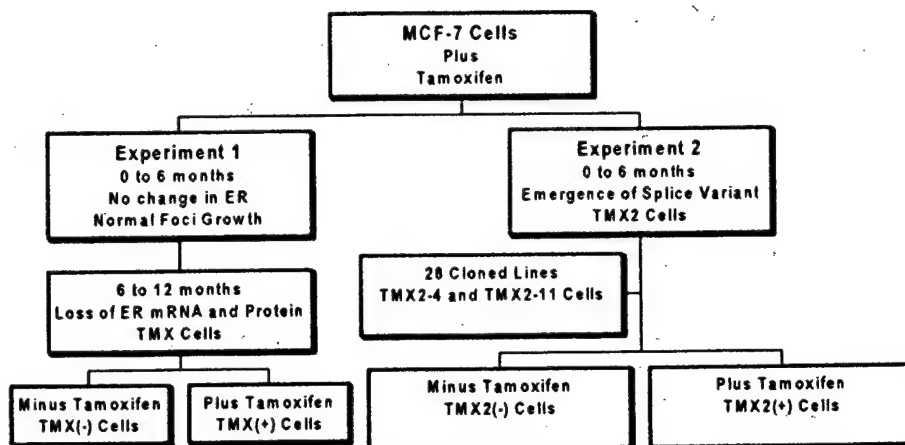
Figure 2
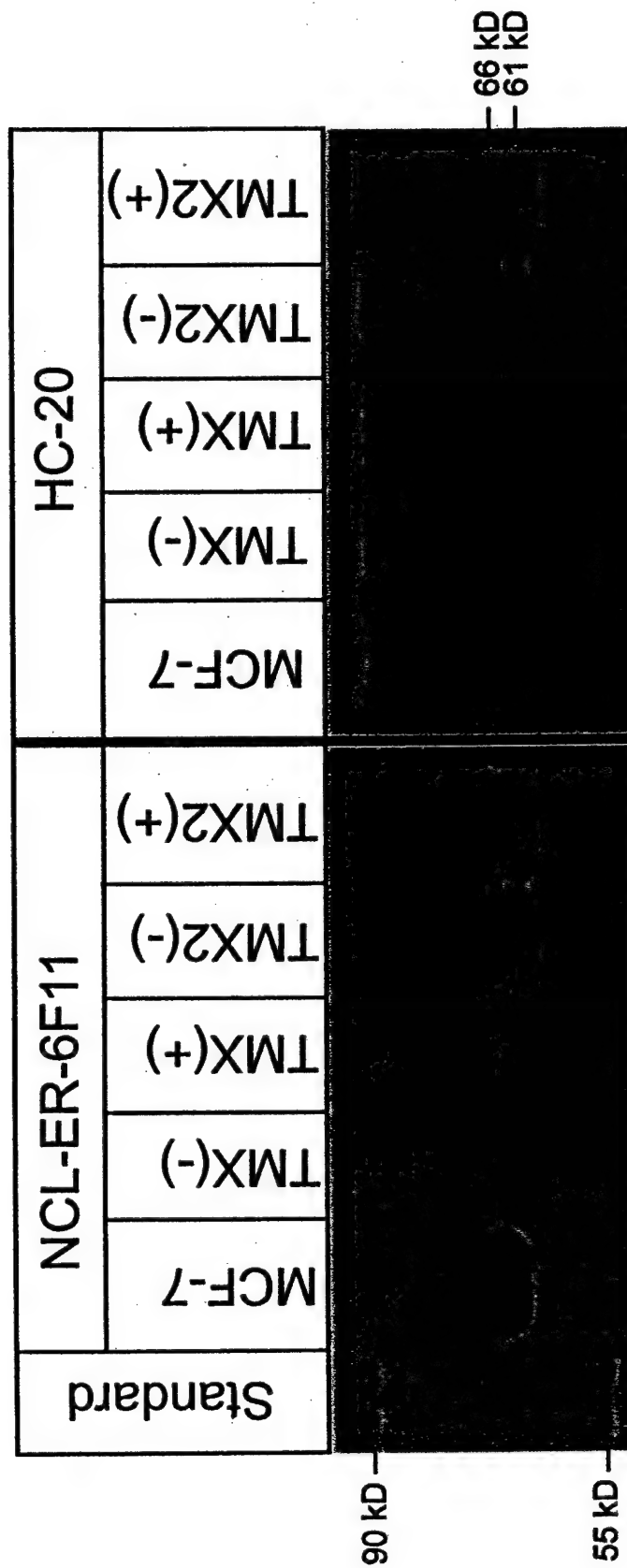
Figure 3

Figure 4

Figure 5

Figure 6

Figure 7